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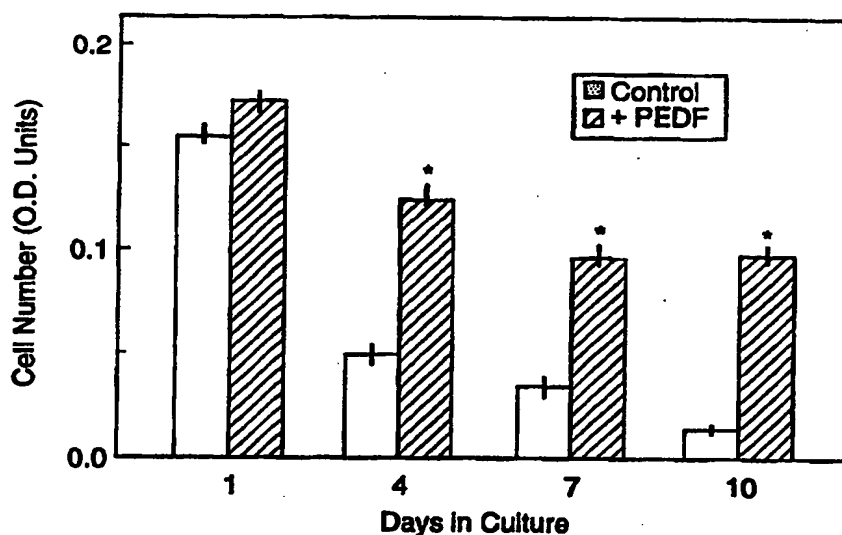
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(54) Title: PIGMENT EPITHELIUM-DERIVED FACTOR: CHARACTERIZATION, GENOMIC ORGANIZATION AND SEQUENCE OF THE PEDF GENE



## (57) Abstract

Nucleic acids encoding the neurotrophic protein known as pigment epithelium-derived factor (PEDF), a truncated version of PEDF referred to as rPEDF, and equivalent proteins, vectors comprising such nucleic acids, host cells into which such vectors have been introduced, recombinant methods for producing PEDF, rPEDF, and equivalent proteins, the rPEDF protein and equivalent proteins of rPEDF and PEDF-BP, -BX and BA, and the PEDF protein produced by recombinant methods. Effects and use of these variants on: 1) neuronal differentiation (neurotrophic effect), 2) neuron survival (neuronotrophic effect), and 3) glial inhibition (gliastatic effect) are described.

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Pigment Epithelium-Derived Factor:

Characterization, Genomic Organization  
and Sequence of the PEDF gene

5 This application is a continuation-in-part of application Serial No. 08/257,963 filed on June 07, 1994, which is a continuation-in-part of application Serial No. 07/952,796 filed on September 24, 1992.

TECHNICAL FIELD OF THE INVENTION

10 This invention relates to a neurotrophic, neuronotrophic and gliastatic protein. More specifically, this invention relates to the biological properties of a protein known as pigment epithelium-derived factor (PEDF) and recombinant forms of the protein. This invention also relates to a truncated version of PEDF that is referred to as rPEDF. In addition to PEDF and rPEDF and functionally  
15 equivalent proteins, this invention relates to nucleic acids that encode rPEDF, and fragments thereof, to vectors comprising such nucleic acids, to host cells into which such vectors have been introduced, and to the use of these host cells to produce such proteins.

20 BACKGROUND OF THE INVENTION

Pigment epithelium-derived factor, otherwise known as pigment epithelium differentiation-factor, was identified in the conditioned medium of cultured fetal human retinal pigment epithelial cells as an extracellular  
25 neurotrophic agent capable of inducing neurite outgrowth in cultured human retinoblastoma cells (Tombran-Tink et al. (1989) *Invest. Ophthalmol. Vis. Sci.*, 30 (8), 1700-1707). The source of PEDF, namely the retinal pigment epithelium (RPE), may be crucial to the normal development and function of the neural retina. A variety of  
30 molecules, including growth factors, are synthesized and secreted by RPE cells. Given that the RPE develops prior to and lies adjacent to the neural retina, and that it functions as part of the blood-retina barrier (Fine et al. (1979) The Retina, Ocular Histology: A Text and Atlas, New  
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York, Harper & Row, 61-70), the RPE has been implicated in vascular, inflammatory, degenerative, and dystrophic diseases of the eye (Elner et al. (1990) *Am. J. Pathol.*, 136, 745-750). In addition to growth factors, nutrients and metabolites are also exchanged between the RPE and the retina. For example, the RPE supplies to the retina the well-known growth factors PDGF, FGF, TGF- $\alpha$ , and TGF- $\beta$  (Campochiaro et al. (1988) *Invest. Ophthalmol. Vis. Sci.*, 29, 305-311; Plouet (1988) *Invest. Ophthalmol. Vis. Sci.*, 29, 106-114; Fassio et al. (1988) *Invest. Ophthalmol. Vis. Sci.*, 29, 242-250; Connor et al. (1988) *Invest. Ophthalmol. Vis. Sci.*, 29, 307-313). It is very likely that these and other unknown factors supplied by the RPE influence the organization, differentiation, and normal functioning of the retina.

In order to study and determine the effects of putative differentiation factors secreted by the RPE, cultured cells have been subjected to retinal extracts and conditioned medium obtained from cultures of human fetal RPE cells. For example, U.S. Patent No. 4,996,159 (Glaser) discloses a neovascularization inhibitor recovered from RPE cells that is of a molecular weight of about 57,000 +/- 3,000. Similarly, U.S. Patent Nos. 1,700,691 (Stuart), 4,477,435 (Courtois et al.), and 4,670,257 (Guedon born Saglier et al.) disclose retinal extracts and the use of these extracts for cellular regeneration and treatment of ocular disease. Furthermore, U.S. Patent Nos. 4,770,877 (Jacobson) and 4,534,967 (Jacobson et al.) describe cell proliferation inhibitors purified from the posterior portion of bovine vitreous humor.

PEDF only recently has been isolated from human RPE as a 50-kDa protein (Tombran-Tink et al. (1989) *Invest. Ophthalmol. Vis. Sci.*, 29, 414; Tombran-Tink et al. (1989) *Invest. Ophthalmol. Vis. Sci.*, 30, 1700-1707; Tombran-Tink et al. (1991) *Exp. Eye Res.*, 53, 411-414).

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Specifically, PEDF has been demonstrated to induce the differentiation of human Y79 retinoblastoma cells, which are a neoplastic counterpart of normal retinoblasts (Chader (1987) *Cell Different.*, 20, 209-216). The differentiative changes induced by PEDF include the extension of a complex meshwork of neurites, and expression of neuronal markers such as neuron-specific enolase and neurofilament proteins. This is why the synthesis and secretion of PEDF protein by the RPE is believed to influence the development and differentiation of the neural retina. Furthermore, PEDF is only highly expressed in undifferentiated human retinal cells, like Y79 retinoblastoma cells, but is either absent or downregulated in their differentiated counterparts. Recently, it was reported that PEDF mRNA is expressed in abundance in quiescent human fetal W1 fibroblast cells and not expressed in their senescent counterparts (Pignolo et al., 1993).

Further study of PEDF and examination of its potential therapeutic use in the treatment of inflammatory, vascular, degenerative, and dystrophic diseases of the retina and central nervous system (CNS) necessitates the obtention of large quantities of PEDF. Unfortunately, the low abundance of PEDF in fetal human eye and furthermore, the rare availability of its source tissue, especially in light of restrictions on the use of fetal tissue in research and therapeutic applications, make further study of PEDF difficult at best. Therefore, there remains a need for large quantities of PEDF and equivalent proteins. Accordingly, the obtention of nucleic acids that encode PEDF and equivalent proteins, and the capacity to produce PEDF and equivalent proteins in large quantities would significantly impact upon the further study of PEDF, its structure, biochemical activity and cellular function, as well as the discovery and design of therapeutic uses for PEDF.

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SUMMARY OF THE INVENTION

It is an object of the present invention to provide nucleic acids encoding for PEDF and functional fragments thereof, vectors comprising such nucleic acids, host cells into which such vectors have been introduced, and a recombinant method of producing PEDF and equivalent proteins. It is another object of the present invention to obtain the genomic DNA sequences encoding for PEDF, identify the intron-exon junctions, the chromosome location in the human genome, and to provide the regulatory regions of the gene which flank the genomic sequence. The present invention relates to such genomic PEDF DNA.

It is a further object of the present invention to provide structural characteristics of PEDF and its similarities to the serpin family of serine protease inhibitors, both structural and functional.

It is yet another object of the present invention to provide PEDF and equivalent proteins produced in accordance with such a recombinant method, wherein the PEDF and equivalent proteins so produced are free from the risks associated with the isolation of PEDF from naturally-occurring source organisms.

Another object of the present invention is to provide nucleic acids for a truncated version of PEDF, referred to as rPEDF, and equivalent proteins, vectors comprising such nucleic acids, host cells into which such vectors have been introduced, and a recombinant method of producing rPEDF and equivalent proteins. It is also an object of the present invention to provide rPEDF and equivalent proteins produced in accordance with such a recombinant method.

It is a further object of the invention to provide a PEDF protein having neuronotrophic and gliastatic activity. The neuronotrophic activity is seen in the prolonged survival of neuronal cells. The

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gliastatic activity is observed in the inhibition of growth of glial cells in the presence of PEDF or active fragment thereof. It is another object of the invention to provide methods for treating neuronal cells so as to promote/enhance neuron survival and prevent growth of glial cells, comprising treating such cell populations with an effective amount of PEDF or an active fragment thereof.

It is yet another object of the present invention to provide antibodies which specifically recognize PEDF, either monoclonal or polyclonal antibodies, raised against native protein, the recombinant protein or an immunoreactive fragment thereof. It is an object of the invention to provide methods for detecting PEDF by immunoassay using such antibody preparation in determining aging and/or other degenerative diseases. Another object of the invention relates to a method of using PEDF antibodies to specifically inhibit PEDF activity.

These and other objects and advantages of the present invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

#### Descriptions of the Figures

Figure 1: Human PEDF Gene Structure:  
Restriction map and organization of the human PEDF gene. Exons 1-8 are indicated by black boxes and numbered 1-8. Introns and flanking DNA are represented by horizontal line and are labeled A-G. Positions of several genomic clones are shown above and below the diagrammed gene. Recognition sites for the restriction endonuclease, NotI ("N"), BamHI ("B") and EcoRI ("E") are indicated by vertical arrows.

Figure 2: Southern analysis of human genomic DNA (A) and P147 (B) restricted with Bam HI, EcoRI, HindIII and PstI endonuclease. Southern membranes from

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- ° Pulsed-field electrophoretic gel profiles were probed with radioactively labelled PEDF cDNA. The pattern of hybridization of P147 DNA is consistent with total human genomic DNA. Size markers are indicated.

Figure 3: 5' Flanking region of the PEDF gene. The first exon (capital letters) and the first 1050 bp of 5 prime flanking region are shown. Two Alu repetitive sequences are underlined. Possible binding sites for HNF-1, PEA3, Octomer (Oct), c/EBP are underlined and labeled. The putative AP-1 sites are shown in bold, and TREp/RAR are double underlined. The underlined (dashed) sequence in exon 1 was determined by the 5' RACE.

Figure 4: Northern Blot analysis of PEDF mRNA: Gene expression analysis of the human PEDF transcript in a number of human adult and fetal tissues. Tissues from which RNA was obtained are shown above corresponding lanes. Membranes contain 2 ug poly (A) RNA for each sample and were probed with radioactively labelled cDNA for human PEDF. A single 1.5 kb transcript is seen in both adult and fetal tissues with the greatest intensity of hybridization in liver, testis, skeletal muscle and ovary while the signal for brain, pancreas and thymus was significantly weaker than that for other tissues. No significant signal was detected for adult kidney and spleen. A significant difference in PEDF mRNA levels seen between adult and fetal kidney.

Figure 5: Evolutionary relatedness of the Human PEDF gene: Each lane represents a total of 8 ug of genomic DNA for each species digested with Eco RI. Southern blot analysis is shown with a PEDF probe. Hybridization signals for chicken (A), mammals (B) and primates (C) is shown. A large fragment of approximately 23 kb is seen in all primates and many mammalian species. In addition several polymorphisms are seen in the different mammalian species examined.



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Figure 6A & 6B: Relationship between cell density plated and optical density measured by MTS assay. Different concentrations of postnatal-day 8 cerebellar granule cells were added to 96 well plate and cultured in serum-containing medium (6A), or chemically defined medium (6B). Optical density was measured on days in vitro (DIV) 1, 4, or 7. Square, DIV 1; Solid circle, DIV 4; Open circle, DIV7. The data are plotted as function of cell density (n=6).

Figure 7: Time course for PEDF stimulation of cell survival in chemically-defined medium. Postnatal-day 8 cerebellar granule cells were cultured in 96 well plate. PEDF was added at DIV 0 and the optical density was then measured on DIV 1, 4, 7, or 10. Solid bar, control; cross-hatched bar, PEDF treated (50ng/ml); striped bar, PEDF treated (500ng/ml). The data are expressed as optical density/well (means $\pm$ SEM, n=6). Statistical analysis was done by two way ANOVA post-hoc Scheefe test. \*\*P<0.0001 versus control.

Figure 8: Dose-response curve for PEDF in chemically defined medium. Different concentrations of PEDF were added on DIV 0 and MTS assay was carried out on DIV 7. The data are expressed as ratio to control (mean  $\pm$  SEM, n=6). Statistical analysis was done by one way ANOVA post-hoc Scheffe F test. \*\*P<0.0001 vesus control.

Figure 9: MTS assay of postnatal day 5 cerebellar granule cells at DIV 1 and DIV 2. Postnatal-day 5 cerebellar granule cells were cultured in 96 well plate using serum-containing medium without Ara-C (A), or chemically defined medium without F12(B). The MTS assay was carried out on DIV 1 and 2. Solid bar, control; Striped bar, PEDF treated (500ng/ml). The data are expressed as optical density/well (means  $\pm$  SEM, n=6). Statistical analysis was done by two way ANOVA post-hoc Scheffe F test. \*\*P<0.0005 vesus control.

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Figure 10: BrdU incorporation into postnatal day 5 cerebellar granule cells. Postnatal-day 5 cerebellar granule cells were cultured in a 96 well plate using serum-containing medium (SCM) without Ara-C, or chemically defined medium (CDM) without F12. PEDF was added on DIV 0, BrdU was added on DIV 1 and the cells were fixed on DIV 2. Solid bar, control; Striped bar, PEDF treated (500ng/ml). The number of labeled nucleic acids are expressed as a percentage of total cell population (mean  $\pm$  SEM). For each value, 3000 cells was counted at least.

Figure 11: Relationship between cell density and neurofilament content measured by ELISA. Different concentrations of postnatal-day 8 cerebellar granule cells are added to 96 wells and cultured. Optical density was measured on DIV 7. The data are plotted as a function of cell density.

Figure 12: Neurofilament ELISA assay in postnatal-day 8 cerebellar granule cells. Cells were cultured in a 96 well plate with or without PEDF using serum-containing medium (SCM) or chemically defined medium (CDM). After fixing cells on DIV 7, the neurofilament ELISA was carried out and the data are expressed as ratio to control (mean  $\pm$  SEM, n=6 to 10). Solid bar, control; Striped bar, PEDF treated (500ng/ml). Statistical analysis was done by two way ANOVA post-hoc Scheffe F test. \*P <0.05 vesus control.

Figure 13: Summary of PEDF neuronotrophic effects through 10 days in culture.

Figure 14: Effects of truncated peptides BP and BX on CGC viability.

Figure 15: Effect of PEDF on astroglia from cerebellum.

Figure 16: Effect of PEDF on cerebellar microglia.

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Figure 17: Purification of PEDF-immunoreactive protein from bovine IPM. Washes of bovine IPM were subjected to A) TSK-3000 size-exclusion chromatography followed by B) Mono-S chromatography. Western blot inserts demonstrate the fractions containing PEDF.

Figure 18: Enzymatic deglycosylation of PEDF as demonstrated by Western blotting. PEDF treatment is given at the top of each lane. Numbers indicate positions of mol. wt. standards.

Figure 19: Antibody to rPEDF specifically recognizes native PEDF at a high titer. A) Western blot demonstrating effectiveness of the antibody to at least 1:50,000 dilution and that addition of excess rPEDF completely blocks band visualization. B) Slot-blot analysis shows the ability to detect  $\leq 1$  ng of native bovine PEDF protein.

Figure 20: Negative effect of PEDF antibody on neurite extension in Y-79 cells. Top row: bovine serum albumin (BSA) control cultures. Middle row: antibody effect on neurite-induction by native bovine PEDF protein. Bottom row: antibody effect on neurite induction by interphotoreceptor matrix (IPM).

Figure 21: Phase microscopy analysis of neurite outgrowth in the presence or absence of PEDF.

Figure 22: Phase microscopy analysis of neurite outgrowth in the presence of recombinant PEDF and native, isolated PEDF.

Figure 23: Schematic Diagram of C-terminal deletions of rPEDF.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a protein having novel, important and unobvious properties. Pigment epithelium-derived factor (PEDF) is a protein having neurotrophic, neuronotrophic and gliastatic characteristics. The present invention further relates to the DNA sequences coding for the PEDF gene, the genomic

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° DNA containing the PEDF gene and fragments of the PEDF gene encoding for protein fragments of PEDF having biological activity.

"Neurotrophic" activity is defined herein as the ability to induce differentiation of a neuronal cell population. For example, PEDF's ability to induce differentiation in cultured retinoblastoma cells is considered neurotrophic activity.

"Neuronotrophic" activity is defined herein as the ability to enhance survival of neuronal cell populations. For example, PEDF's ability to act as a neuron survival factor on neuronal cells is neuronotrophic activity.

"Gliastatic" activity is defined herein as the ability to inhibit glial cell growth and proliferation. For example, PEDF's ability to prevent growth and/or proliferation of glial cells is gliastatic activity.

Based upon the protein amino acid sequence elucidated in the present invention, PEDF has been found to have extensive sequence homology with the serpin gene family, members of which are serine protease inhibitors. Many members of this family have a strictly conserved domain at the carboxyl terminus which serves as the reactive site of the protein. These proteins are thus thought to be derived from a common ancestral gene. However the developmental regulation differs greatly among members of the serpin gene family and many have deviated from the classical protease inhibitory activity (Bock (1990) Plenum Press, New York Bock, S.C., *Protein Eng.* 4, 107-108; Stein et al. (1989) *Biochem. J.* 262, 103-107). Although PEDF shares sequence homology with serpins, analysis of the cDNA sequence indicates that it lacks the conserved domain and thus may not function as a classical protease inhibitor.

Genomic sequencing and analysis of PEDF has provided sequences of introns and exons as well as

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approximately 4 kb of 5'-upstream sequence. The present invention demonstrates the localization of the gene for PEDF to 17p13.1 using both in situ hybridization and analyses of somatic cell hybrid panels (Tombran-Tink, et al., (1994) *Genomics*, 19:266-272). This is very close to the p53 tumor suppressor gene as well as to the chromosomal localization of a number of hereditary cancers unrelated to mutations in the p53 gene product. PEDF thus becomes a prime candidate gene for these cancers.

The full length genomic PEDF sequence is represented by SEQ ID NO:43. The PEDF gene encompasses approximately 16 Kb and contains 8 exons all of which have conventional consensus splice-sites. The 5' flanking region of the PEDF gene contains two Alu repetitive elements which cover approximately two thirds of the first 1050 bp of the putative promoter sequence. There are also several sequence motifs which may be recognized by members of several families of transcription factors. The presence of two possible binding sites for the ubiquitous octamer family of transcription factors, may explain the presence of PEDF in most tissues tested. The presence of other more specific elements, however, suggests that PEDF is under precise control and supports previous work including its effects on such diverse processes as neuronal differentiation and fibroblast senescence.

The genomic PEDF sequence or fragments thereof are useful as a probe for detecting the gene in a cell. In addition, such a probe is useful in a kit for identification of a cell type carrying the gene. Mutations, deletions or other alternations in the gene organization can be detected through the use of a DNA probe derived from the PEDF genomic sequence.

#### Tissue Distribution

Although PEDF is particularly highly expressed by RPE cells, it is detectable in most tissues, cell types, tumors, etc. by Northern and Western blot analyses.

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° It is readily detected, for example in vitreous and aqueous humors. The important question of subcellular localization of PEDF has also been addressed. Although the bulk of the PEDF appears to be secreted, we have used a PEDF antibody to probe cultured monkey RPE cells and found that PEDF is associated with the nucleus as well as with very specific cytoskeletal structures in the cytoplasm. Importantly, this varies as to the age of the cells and the specific cell-cycle state examined. For example, the protein appears to concentrate at the tips of the pseudopods of primate RPE cells that interact with the substratum during the initial stages of attachment. Later though, this staining disappears and there is appearance of the protein in association with specific cytoskeletal structures and the nucleus. Thus it appears that PEDF plays an important intracellular role in both nucleus and cytoplasm.

#### Involvement in Cell Cycle

The present invention indicates that there is expression in dividing, undifferentiated Y-79 cells and little or no expression in their quiescent, differentiated counterparts (Tombran-Tink, et al. (1994) *Genomics*, 19:266-272).. Pignolo et al. (1993) *J. Biol. Chem.*, 268:2949-295) have demonstrated that the synthesis of PEDF in WI-38 fibroblast cells is restricted to the G<sub>0</sub> stage of the cell cycle in young cells. Moreover, in old senescent cells, PEDF messenger RNA is absent.

#### Production of Recombinant PEDF.

Segmentation of the PEDF polypeptide is basic to studies on structure-function. For this purpose, expression vectors containing fragments of PEDF coding sequences provide an excellent source for synthesizing and isolating different regions of the PEDF polypeptide. Expression of human fetal PEDF sequences was achieved with *E. coli* expression vectors and the human fetal PEDF cDNA. We have shown that the recombinant PEDF product (rPEDF) is

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° a biologically-active neurotrophic factor and is obtained  
in yields on the order of 1.3 mg/g of wet E. coli cells.  
Truncated peptides can also be made from appropriate  
molecular biological constructs and expressed in E. coli.  
Using these products, we have evidence that two distinct  
5 regions on the PEDF primary structure can be  
distinguished: 1) an "active site" conferring neurotrophic  
activity on the molecule that is located within amino acid  
residues 44-121 near the N-terminal of the protein and 2)  
10 a region near the C-terminal with homology to a serpin  
exposed loop i.e., the "classical" serpin active site.  
These results suggest 1) that the overall native  
conformation of PEDF is not required for neurite outgrowth  
and 2) that inhibition of serine proteases can not account  
for the biological activity of PEDF. We now have a series  
15 of truncated rPEDF constructs that span the protein  
sequence and can pinpoint the specific neurotrophic  
"active site" near the N-terminal.

Characterization with a highly  
specific polyclonal antibody.

20 Purified recombinant human PEDF was used to  
develop a polyclonal antibody ("Anti-rPEDF") that  
specifically blocks the PEDF-mediate neurotrophic  
activity. Furthermore, the anti-rPEDF completely blocks  
the IPM-induced neurotrophic activity.

25 Neuronotrophic properties of PEDF

In addition to demonstrating that native PEDF  
and rPEDF are neurotrophic in the Y-79 and Weri tumor cell  
systems, the present invention determined whether PEDF had  
an effect on normal neurons in primary culture. For this  
30 purpose, studies were conducted using cultures of normal  
cerebellar granule cells (CGCs) prepared from the 8-day  
postnatal rat. Cells treated with rPEDF did not respond  
to treatment by exhibiting a more neuronal morphological  
appearance. However, PEDF had a large effect on granule  
35 cell survival. Since these cells are not tumorous or

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transformed cells, they have a finite life in culture, dying in about 21 days depending on the culture medium. PEDF-treated culture, however, contained up to 10-fold more cells after 10 days of culture in serum-free medium compared to non-treated culture (Figure 4). These results were determined; 1) by direct microscopic observation and cell counting and 2) use of an MTS (tetrazolium/formazan) assay which determines live cell numbers (See example 11). Thus, PEDF has a dramatic effect on CNS neuron survival and should be added to the short list of newly-emerging "neuronotrophic" proteins.

In General Tissue Culture Research:

Two problems that generally plague any tissue culture experiment using neurons and glia is that the neurons tend to die quickly and that glia tend to overrun the culture dish. PEDF or its peptides can help in both regards. Thus, one commercial use of PEDF might be as a general culture medium additive when CNS cells are to be cultured.

In CNS Transplantation Studies:

It is thought that transplantation of neurons may cure certain pathologies. For example, in Parkinson's disease, transplantation of specific fetal brain cells into patients could alleviate or cure the problems associated with the disease. One of the major problems to contend with, though, would be to prolong the life of the transplanted cells and to keep them differentiated, e.g. secreting the proper substances, etc. Pretreatment of the cells with PEDF could aid in both of these areas. Similarly, transfection of either neurons or astroglia with the PEDF gene before implantation can be a long-term source of PEDF at the transplantation site.

There is much activity in attempts at transplantation of neural retina and photoreceptor cells to help cure blindness. Attempts to date have not been fruitful both due to non-differentiation and death of the



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grafts. Again, PEDF may help in both regards. Specifically, photoreceptor neurons to be transplanted can be pretreated with PEDF or the gene transfected into the cells before surgery. Alternatively, PEDF can be transfected at high levels into adjacent retinal pigment epithelial (RPE) cells where they can serve as a supranormal source of the protein. Several investigators have now shown that cultured RPE cells survive very well after transplantation into the interphotoreceptor space of test animals. Transfection of human RPE cells *in vitro* with the PEDF gene then use of them in retinal transplantation thus is feasible.

#### In Neurodegenerative Diseases:

Many neurodegenerative diseases and other insults to the CNS (brain and retina) are typified by death of neurons and overpopulation by glia (gliosis). PEDF can be used effectively in these conditions to prolong the life and functioning of the primary neurons and to stave off the glial advance. PEDF can be effective, for example, in blocking microglial activation in response to CNS injury as well as prolonging/sparing the lives of neurons.

In the retina, it is predictable that PEDF inhibits the Muller glial cells. Since Muller cells are similar to astroglia, PEDF would be similarly effective in blocking gliosis in conditions such as retinal detachment, diabetes, Retinitis Pigmentosa, etc. as well as sparing the lives of the retinal neurons.

#### In Glial Cancers:

Most of the major forms of cancer that strike the CNS involve glial elements, PEDF is a gliastatic factor that can be used in combination with other forms of therapy. For example, along with surgery, PEDF can effectively inhibit the spread or reoccurrence of the disease.

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Genetic Analysis

The present invention relates to the determination of the organization of the human PEDF gene and its promoter and analysis of its evolutionary relatedness and expression in a variety of human fetal and adult tissues.

The present invention provides, among other things, a nucleic acid which encodes PEDF. In particular, a cDNA sequence is provided as set forth in SEQ ID NO:1. This cDNA sequence codes for PEDF, which has the amino acid sequence set forth in SEQ ID NO:2. Further genomic sequences are mapped in figure 1 and provided SEQ ID NO:43. Additional fragments of the genomic PEDF sequence are provided in SEQ ID NO: 9 through SEQ ID NO: 12. The location of intron-exon junctions are identified in table 1 and SEQ ID NO: 25 through SEQ ID NO: 40 and SEQ ID NO:43.

The term "nucleic acid" refers to a polymer of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), which can be derived from any source, can be single- or double-stranded, and can optionally contain synthetic, non-natural, or altered nucleotide which are capable of being incorporated into DNA or RNA polymers. The nucleic acid of the present invention is preferably a segment of DNA.

The present invention further provides truncated versions of PEDF. The largest of these is referred to as rPEDF, and comprises the amino acid sequence Met-Asn-Arg-Ile fused to Asp<sup>44</sup>...Pro<sup>418</sup> of PEDF, the amino terminus of which has been deleted. The rPEDF protein comprises the amino acid sequence of SEQ ID NO:3. The present invention also provides a nucleic acid which encodes a protein comprising the amino acid sequence of rPEDF, i.e., the amino acid sequence of SEQ ID NO:3.

One who is skilled in the art will appreciate that more than one nucleic acid may encode any given

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protein in view of the degeneracy of the genetic code and the allowance of exceptions to classical base pairing in the third position of the codon, as given by the so-called "Wobble rules". Accordingly, it is intended that the present invention encompass all nucleic acids that encode the amino acid sequences of SEQ ID NO:2 and SEQ ID NO:3, as well as equivalent proteins. The phrase "equivalent nucleic acids" is intended to encompass all of these nucleic acids.

It also will be appreciated by one skilled in the art that amino acid sequences may be altered without adversely affecting the function of a particular protein. In fact, some alterations in amino acid sequence may result in a protein with improved characteristics. The determination of which amino acids may be altered without adversely affecting the function of a protein is well within the ordinary skill in the art. Moreover, proteins that include more or less amino acids can result in proteins that are functionally equivalent. Accordingly, it is intended that the present invention encompass all amino acid sequences that result in PEDF protein or functional protein fragments thereof.

Some examples of possible equivalent nucleic acids and equivalent proteins include nucleic acids with substitutions, additions, or deletions which direct the synthesis of the rPEDF protein and equivalent protein fragments thereof; nucleic acids with different regulatory sequences that direct the production of rPEDF proteins; variants of rPEDF which possess different amino acids and/or a number of amino acids other than four fused to the amino terminal end of the protein; and PEDF and rPEDF and functional protein fragments thereof with amino acid substitutions, additions, deletions, modifications, and/or posttranslational modifications, such as glycosylations, that do not adversely affect activity. Since the neurotrophic activity has been correlated to a particular

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° portion of the PEDF protein fragments containing these residues are clearly within the scope of the present invention.

The present invention also provides a vector which comprises a nucleic acid of SEQ ID NO:1, a nucleic acid which encodes a protein comprising the amino acid sequence of SEQ ID NO:2 or an equivalent protein, a nucleic acid which encodes a protein comprising the amino acid sequence of SEQ ID NO:3 or conservatively modified variant proteins, and conservatively modified variant nucleic acids thereof.

In particular, the present invention provides the vector  $\pi$ FS17, which comprises the nucleic acid of SEQ ID NO:1, and the vector pEV-BH, which comprises a nucleic acid which encodes a protein comprising the amino acid sequence of SEQ ID NO:3. It will be appreciated by those skilled in the art that the cDNA inserts described can be present in alternative vectors. For example, inserts can be in vectors of different nature, such as phages, viral capsids, plasmids, cosmids, phagemids, YACs, or even attached to the outside of a phage or viral capsid. The vectors can differ in host range, stability, replication, and maintenance. Moreover, the vectors can differ in the types of control exerted over cloned inserts. For example, vectors can place cloned inserts under the control of a different promoter, enhancer, or ribosome binding site, or even organize it as part of a transposon or mobile genetic element.

The present invention also provides a host cell into which a vector, which comprises a nucleic acid of SEQ ID NO:1, a nucleic acid which encodes a protein comprising the amino acid sequence of SEQ ID NO:2 or an equivalent protein, a nucleic acid which encodes a protein comprising the amino acid of SEQ ID NO:3 or an equivalent protein, or an equivalent nucleic acid thereof, has been introduced. In particular, the host cell may have the vector  $\pi$ FS17,

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° which comprises the nucleic acid of SEQ ID NO:1, or the vector pEV-BH, which comprises a nucleic acid which encodes a protein comprising the amino acid sequence of SEQ ID NO:3.

5 The vectors of the present invention can be introduced into any suitable host cell, whether eukaryotic or prokaryotic. These host cells may differ in their preferred conditions for growth, their nutritive requirements, and their sensitivity to environmental agents. Any appropriate means of introducing the vectors  
10 into the host cells may be employed. In the case of prokaryotic cells, vector introduction may be accomplished, for example, by electroporation, transformation, transduction, conjugation, or mobilization. For eukaryotic cells, vectors may be  
15 introduced through the use of, for example, electroporation, transfection, infection, DNA coated microprojectiles, or protoplast fusion.

The form of the introduced nucleic acid may vary with the method used to introduce the vector into a host  
20 cell. For example, the nucleic acid may be closed circular, nicked, or linearized, depending upon whether the vector is to be maintained as an autonomously replicating element, integrated as provirus or prophage, transiently transfected, transiently infected as with a  
25 replication-disabled virus or phage, or stably introduced through single or double crossover recombination events.

The present invention also provides a method of producing PEDF, rPEDF, and equivalent proteins, which method comprises expressing the protein in a host cell.  
30 For example, a host cell into which has been introduced a vector which comprises a nucleic acid of SEQ ID NO:1, a nucleic acid which encodes a protein comprising the amino acid sequence of SEQ ID NO:2 or an equivalent protein, a nucleic acid which encodes a protein comprising the amino  
35 acid of SEQ ID NO:3 or an equivalent protein, or an

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° equivalent nucleic acid thereof, may be cultured under suitable conditions to produce the desired protein. In particular, a host cell into which has been introduced the vector  $\pi$ FS17, which comprises the nucleic acid of SEQ ID NO:1, or the vector pEV-BH, which comprises a nucleic acid  
5 which encodes a protein comprising the amino acid sequence of SEQ ID NO:3, may be cultured under suitable conditions to produce the proteins comprising the amino acid sequences of SEQ ID NO:2 and SEQ ID NO:3, respectively.

The present invention also provides  
10 recombinantly produced PEDF, and functional protein fragments thereof which have been produced in accordance with the aforementioned present inventive method of culturing an appropriate host cell to produce the desired protein. The production of a protein such as PEDF by  
15 recombinant means enables the obtention of large quantities of the protein in a highly purified state, free from any disease-causing agents which may accompany the protein isolated or purified from a naturally occurring source organism, and obviates the need to use, for  
20 example, fetal tissue as a source for such a protein.

Recombinant PEDF and functional protein fragments thereof may be supplied as active agents to cells by a variety of means, including, for example, the introduction of nucleic acids, such as DNA or RNA, which  
25 encode the protein and may be accordingly transcribed and/or translated within the host cell, the addition of exogenous protein, and other suitable means of administration as are known to those skilled in the art. In whatever form in which supplied, the active agent can  
30 be used either alone or in combination with other active agents, using pharmaceutical compositions and formulations of the active agent which are appropriate to the method of administration. Pharmaceutically acceptable excipients, i.e., vehicles, adjuvants, carriers or diluents, are well-  
35 known to those who are skilled in the art, and are readily

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available. The choice of excipient will be determined in part by the particular compound, as well as by the particular method used to administer the compound. Accordingly, there is a wide variety of suitable formulations which can be prepared in the context of the present invention. However, pharmaceutically acceptable excipients not altering the neurotrophic, neuronotrophic and gliastatic activities of the recombinant protein are preferred.

The following examples serve to illustrate further the present invention and are not to be construed as limiting its scope in any way.

#### EXAMPLE 1

This example describes the trypsin digestion of PEDF and the amino acid sequencing of the resulting fragments.

PEDF was purified from the medium of a primary culture of human fetal RPE cells by high performance liquid chromatography (HPLC). The HPLC-purified PEDF was then reduced and alkylated. Afterwards, it was dried and redissolved in 50  $\mu$ l of CRA buffer (8 M urea, 0.4 M ammonium carbonate, pH 8.0), and 5  $\mu$ l of 45 mM dithiothreitol (DTT) (Calbiochem, San Diego, CA) were added. After heating at 50°C for 15 minutes, the solution was cooled, and 5  $\mu$ l of 100 mM iodoacetic acid (Sigma Chem. Co., St. Louis, MO) were added. After 15 minutes, the solution was diluted to a concentration of 2 M urea and subjected to trypsin digestion (Boehringer-Mannheim, Indianapolis, IN) for 22 hours at 37°C using an enzyme:substrate ratio of 1:25 (wt/wt). Tryptic peptides were separated by narrowbore, reverse-phase HPLC on a Hewlett-Packard 1090 HPLC, equipped with a 1040 diode array detector, using a Vydac 2.1 mm X 150 mm C18 column. A gradient of 5% B at 0 minutes, 33% B at 63 minutes, 60% B at 95 minutes, and 80% B at 105 minutes, with a flow rate of 150  $\mu$ l/minute, was used. In this gradient, buffer

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A was 0.06% trifluoroacetic acid/H<sub>2</sub>O, and buffer B was 0.055% trifluoroacetic acid/acetonitrile. Chromatographic data at 210 and 277 nm, and UV spectra from 209 to 321 nm, of each peak were obtained. Samples for amino-terminal sequence analysis were applied to a polybrene precycled glass fiber filter and subjected to automated Edman degradation (Harvard Microchemical Facility, Boston, MA) on an ABI model 477A gas-phase protein sequencer (program NORMAL 1). The resulting phenylthiohydantoin amino acid fractions were manually identified using an on-line ABI Model 120A HPLC and Shimadzu CR4A integrator.

Trypsin digestion of purified PEDF and amino acid analysis of the resulting fragments yielded nonoverlapping peptide sequences, including the sequences JT-3 (SEQ ID NO:6):

Thr Ser Leu Glu Asp Phe Tyr Leu Asp Glu Glu Arg  
 1 5 10  
 Thr Val Arg Val Pro Met Met  
 15

and JT-8 (SEQ ID NO:7):

Ala Leu Tyr Tyr Asp Leu Ile Ser Ser Pro Asp Ile  
 1 5 10  
 His Gly Thr Tyr Lys Glu Leu Leu Asp Thr Val Thr  
 15 20  
 Ala Pro Gln Xaa Asn  
 25

25

## EXAMPLE 2

This example describes the construction of oligonucleotides, based on the peptide sequences of Example 1, the use of the oligonucleotides in the isolation of PEDF cDNA, and the sequencing of PEDF cDNA.

Based on the JT-3 and JT-8 peptide sequences of Example 1 and codon usage data, the oligonucleotides oFS5665 (SEQ ID NO:4): 5'-AGYAAATTTYTAYGAYCTSTA-3' and oFS5667 (SEQ ID NO:5): 5'-CTYTCYTCRTCSAGRTARAA-3' were

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constructed on an ABI 392 DNA/RNA Synthesizer and used as primers in a polymerase chain reaction (PCR).

A human fetal eye Charon BS cDNA library (obtained from Dr. A. Swaroop of the Kellog Eye Institute) was amplified once (Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)) and screened by PCR (Friedman et al., Screening of  $\lambda$ gt11 Libraries, In: PCR Protocols: A Guide to Methods and Applications, Innis et al., eds., Academic Press, NY (1990), pp. 253-260) using a Techne thermal cycler and standard reagents (GeneAMP, Perkin-Elmer Cetus), except that  $\text{MgSO}_4$  was used at 3 mM. A PCR amplification fragment of about 350 bp was isolated on a 3% NuSieve 3:1 gel (FMC Biochemicals, Rockland, ME) using NA-45 DEAE-cellulose paper (Schleicher and Schuell) (Sambrook et al., supra). The fragment was labeled with  $\alpha^{32}\text{P}$ -dCTP (Amersham Corp., Arlington Heights, IL) by random priming (Random Priming kit, Boehringer-Mannheim, Indianapolis, IN), and used to screen 200,000 plaque-forming units (PFUs) of the human fetal eye library.

Eight positive clones were isolated (Sambrook et al., supra), and DNA of the positive clones was purified according to Qiagen Maxi preparation protocols (Qiagen, Inc., Chatsworth, CA). The inserts of the positive clones were cut out with Not I (BRL, Gaithersburg, MD), circularized with T4 DNA ligase (New England Biolabs, Beverly, MA), transformed into Escherichia coli Epicurian Sure competent cells (Stratagene, Inc., La Jolla, CA), and plated onto Luria broth (LB) plates containing ampicillin and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-gal).

White colonies were selected on the basis that such colonies should possess an insert, and plasmid DNA from single colony cultures were isolated by the Qiagen plasmid miniprep protocol. Purified plasmids were digested with EcoR I and Hind III (BRL). These restriction sites were added during library construction

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through the ligation of linkers to the 5' and 3' ends of the insert, thus EcoR I- Hind III digestion excises the insert present in isolated plasmids. These fragments were electrophoresed on a 0.7% agarose gel to determine insert size. The plasmid possessing the largest insert, namely  $\pi$ FS17, was selected for mapping and subsequent sequencing using the Sequenase 2.0 sequencing kit (United States Biochemical Corp., Cleveland, OH) to confirm the identity of the clone. Sequence analysis was performed using the MacVector software package (International Biotechnologies, Inc.) and the GenBank® Sequence Data Bank (Intelligenetics, Mountain View, CA).

Sequence analysis of  $\pi$ FS17 revealed a base sequence comprising SEQ ID NO:1, with a long, open reading frame (ORF) encoding the 418 amino acids of SEQ ID NO:2, a typical ATG start codon, and a polyadenylation signal (not shown in SEQ ID NO:1). The coding sequence of the clone aligns exactly with all previously determined PEDF peptide sequences. The deduced amino acid sequence also contains a stretch of hydrophobic amino acids that could serve as a signal peptide. A comparison of the coding sequence and peptide sequence with the GenBank® Data Bank indicates that PEDF is a unique protein having significant homology to the serpin (serine protease inhibitor) gene family, which includes human  $[\alpha]$ -1-antitrypsin. Although some of the members of this gene family exhibit neurotrophic activity (Monard et al. (1983) *Prog. Brain Res.*, 58, 359-364; Monard (1988) *TINS*, 11, 541-544), PEDF lacks homology to the proposed consensus sequence for the serpin reactive domain.

### EXAMPLE 3

This example describes the construction of an expression vector for the production of recombinant PEDF.

An expression vector was constructed using the plasmid  $\pi$ FS17, which contains the full-length cDNA for human PEDF as described in Example 2. The PEDF coding

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sequence was placed under the control of a bacteriophage lambda P<sub>L</sub> promoter present in the plasmid pEV-vrf2 (Crowl et al., Gene, 38, 31-38 (1985)) to obtain the vector pEV-BH. This was accomplished by obtaining a BamH I-Hind III fragment of  $\pi$ FS17 comprising a portion of the PEDF coding region (namely, nucleotide 245 to 1490 of SEQ ID NO:1),  
5 digesting plasmid pEV-vrf2 with EcoR I-Hind III, rendering both fragments blunt by means of a fill-in reaction at the BamH I and EcoR I ends with DNA polymerase I (Klenow fragment), and ligating the resultant blunt-  
10 ended/compatible-ended fragments to each other. The resultant vector pEV-BH places a distance of 8 nucleotide between the Shine-Dalgarno (SD) sequence and the PEDF coding region. The construct specifies Met-Asn-Arg-Lle-Asp<sup>44</sup>---Pro<sup>418</sup> such that a protein of 379 amino acids, known  
15 as rPEDF, is encoded as indicated in SEQ ID NO:3. The amino acids at the amino terminus of the rPEDF protein do not occur in native PEDF and result from the fusion of nucleic acids during the construction of pEV-BH.

To verify production of the recombinant PEDF  
20 protein by pEV-BH, the plasmid was propagated in E. coli strain RRI (Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), bearing the low copy-number compatible plasmid pRK248cIts that contains a gene for encoding a  
25 temperature-sensitive  $\lambda$ cIAt2 repressor (Bernard et al. (1979) Methods in Enzymology, 68, 482-492). Protein induction was performed as described in Becerra et al. (1991) Biochem., 30, 11707-11719, with the following modifications. Bacterial cells containing pEV-BH were  
30 grown in LB medium containing 50  $\mu$ g/ml ampicillin at 32°C to early logarithmic phase, such that OD<sub>600nm</sub>=0.2. The temperature of the culture was rapidly increased to 42°C by incubating the flask in a 65°C water bath, and the bacteria were subsequently grown at 42°C for 2-3 hours in

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- ° an air-flow incubator at 340 rpm. Aliquots were taken for absorbance readings at 600 nm.

Nascent proteins, synthesized following protein induction, were radiolabeled. After the temperature of the culture had reached 42°C, 150  $\mu$ Ci of L-[<sup>35</sup>S]methionine (1040 Ci/mmol, Amersham Corp., Arlington Heights, IL) were added per ml of culture, and incubation was continued at 42°C for 10 minutes and 30 minutes. Cells were harvested by centrifugation and washed with TEN buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl). <sup>35</sup>S-labeled peptides from total bacterial extracts were resolved and analyzed on SDS-12% PAGE followed by fluorography. A band corresponding to a 42,820 M<sub>r</sub> polypeptide was detected 10 and 30 minutes post-induction. The size obtained for the recombinant protein expressed by pEV-BH matched the expected size for the coding sequence subcloned in pEV-BH. In a similar manner, smaller fragments (BP = 28,000 M<sub>r</sub>; BX = 24,000 M<sub>r</sub>; BA = 9,000 M<sub>r</sub>) can be synthesized and purified. BP peptide includes PEDF amino acids 44 through 269, BX peptide includes PEF amino acids 44 through 227, and BA peptide includes PEDF amino acids 44 through 121.

#### EXAMPLE 4

This example describes the construction of expression vectors containing the full-length PEDF cDNA.

In a manner similar to that described in Example 3 for the construction of pEV-BH, the PEDF ORF of plasmid  $\pi$ FS17 was placed under the control of the bacteriophage lambda P<sub>L</sub> promoter present in the plasmids pRC23 and pEV-vrf1 (Crowl et al. Gene, 38, 31-38 (1985)). This was accomplished by obtaining the SfaN I-Hind III fragment of  $\pi$ FS17 comprising a portion of the PEDF cDNA (namely, nucleotide 107 to 1490 of SEQ ID NO:1), digesting the plasmids with EcoR I-Hind III, rendering the fragments blunt by means of a fill-in reaction at the SfaN I and EcoR I ends with DNA polymerase I (Klenow fragment), and ligating the resultant blunt-ended/compatible-ended

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° fragments to each other. The resulting vectors pRC-SH and pEV-SH place a distance of 14 and 8 nucleotide, respectively, between the SD sequence and the PEDF coding region. The construct pRC-SH encompasses the full-length PEDF ORF, and specifies a PEDF protein of 418 amino acids, with its naturally occurring amino terminus, as set forth in SEQ ID NO: 2. The construct pEV-SH encompasses the full-length PEDF ORF, and specifies a PEDF amino-terminal fusion protein of 425 amino acids, with Met-Asn-Glu-Leu-Gly-Pro-Arg (SEQ ID NO:8) preceding the PEDF sequence of SEQ ID NO:2. These additional amino acids at the amino terminus do not occur in native PEDF, and the codons in pEV-SH specifying these additional amino acids result from the fusion of nucleic acids during the construction of pEV-SH.

15 To verify production of the recombinant proteins specified by the two vectors, the vectors were introduced into E. coli strain RRI [pRK248cIts], and protein induction was performed and monitored by metabolic labeling with <sup>35</sup>S-methionine during induction in a manner similar to that set forth in Example 3. The induced expression of the proteins specified by pRC-SH and pEV-SH had a negative effect on bacterial cell growth. In comparison with bacterial cultures containing the parental plasmids, cultures containing pRC-SH and pEV-SH grew and divided more slowly. This negative effect on bacterial growth correlated with the distance between the initiation codon and the SD, which may suggest that a shorter such distance results in more efficient translation of the recombinant protein. A 46,000 M<sub>r</sub> candidate polypeptide for PEDF was not detected in the media or cell lysates of bacterial cultures containing pRC-SH and pEV-SH. However, a 35,000 M<sub>r</sub> protein was observed in extracts of cultures containing pRC-SH and pEV-SH, but not in extracts of cultures containing parental plasmids. This may indicate that the amino-terminal end of PEDF is protease-sensitive

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and that recombinant full-length PEDF is metabolized in this particular host. Alternatively, failure to observe the anticipated-sized recombinant PEDF proteins may reflect an experimental artifact which could be overcome through the use of alternative expression vectors, hosts, inducible promoters, subcloning sites, methods of recombinant protein isolation or detection, or means of protein induction.

#### EXAMPLE 5

This example describes a method for producing large quantities of recombinantly produced PEDF.

A total of 1 g of *E. coli* cells containing rPEDF was resuspended in 50 ml 20mM Tris-HCl, pH 7.5, 20% sucrose, and 1 mM EDTA. The cells were maintained on ice for 10 minutes, sedimented by centrifugation at 4000 x g, and were resuspended in 50 ml of ice-cold water for 10 minutes. Lysed outer cell walls were separated from spheroplasts by centrifugation at 8000 x g.

The pelleted spheroplasts were resuspended in 10 ml of phosphate buffered saline (PBS) containing 5 mM EDTA, 1 µg/ml pepstatin and 20 µg/ml aprotinin. The suspension was probe-sonicated with a sonicator (Ultrasonics, Inc., model W-225) to lyse the cell membranes. Three bursts at 30 second pulses with a 30 second pause were performed while the sample was immersed in an ice-water bath. RNase TI (1300 units, BRL) and DNase I (500 µg, BRL) were added to the sonicated cell suspension, and the suspension was incubated at room temperature for 10 minutes. This suspension was diluted by the addition of 40 ml of phosphate buffered saline (PBS) containing 5 mM EDTA, 1 µg/ml pepstatin and 20 µg/ml aprotinin, and the crude inclusion bodies were sedimented by centrifugation at 13,000 x g for 30 minutes. The particulate material consisting of inclusion bodies was resuspended in 40 ml of PBS containing 25% sucrose, 5 mM EDTA, and 1% Triton X-100, incubated on ice for 10

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minutes, and centrifuged at 24,000 x g for 10 minutes. The washing step was repeated three times. Finally, the inclusion bodies were resuspended in 10 ml of denaturation buffer containing 50 mM Tris-Cl, pH 8.0, 5 M guanidine-Cl, and 5 mM EDTA. The suspension was probe-sonicated briefly for 5 seconds in an ice-water bath. The resulting suspension was incubated on ice for an additional hour. After centrifugation at 12,000 x g for 30 minutes, the supernatant was added to 100 ml of renaturation buffer containing 50 mM Tris-Cl, pH 8.0, 20% glycerol, 1 mM DTT, 1 µg/ml pepstatin, and 20 µg/ml aprotinin, and stirred gently at 4°C overnight to renature the protein. The soluble and insoluble fractions were separated by centrifugation at 13,500 x g for 30 minutes.

The soluble fraction was further purified by concentrating it to 1 ml using a Centricon 30 microconcentrator (Amicon Div., W.R. Grace & Co., Beverly, MA), and dialyzing it against Buffer A (50 mM sodium phosphate, 1 mM DTT, 20% glycerol, 1 mM EDTA, 1 µg/ml pepstatin, and 1 mM benzamidine) at 4°C for 3 hours. The dialyzed extract was centrifuged at 14,000 rpm in an Eppendorf Centrifuge (Model 5415C) for ten minutes. The supernatant fraction was layered on a S-Sepharose fast-flow (Pharmacia, New Market, NJ) column (1 ml bed volume) pre-equilibrated with buffer A. The column was washed with two column-volumes of buffer A. Finally, recombinant rPEDF was eluted with a step gradient of 50, 100, 150, 200, 300, 400, 500, and 1000 mM NaCl in buffer A. Fractions of 1 ml were collected by gravity flow, and were dialyzed against buffer A. Fraction 300, containing recombinant rPEDF, was stored at -20°C. The recovery in fraction 300 was 50 µg per gram of packed cells, which represents 25% of the total protein.

Most of the rPEDF was recovered from the insoluble fraction by dissolving the fraction in 10 ml of 6M guanidinium-Cl in buffer B (50 mM Tris-Cl, pH 8.0, 1 mM

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DTT, 2 mM EDTA). The solution was centrifuged at 10,000 x g for 5 minutes. The supernatant was layered onto a Superose-12 (Pharmacia, New Market, NJ) column attached in tandem to a second Superose-12 column (each column 2.6 cm x 95 cm) pre-equilibrated with buffer containing 4 M guanidinium-Cl in buffer B. The flow rate was 3 ml/minute. Recombinant rPEDF containing fractions from the Superose-12 column were pooled and dialyzed against buffer C (4 M urea, 50 mM sodium phosphate, pH 6.5, 1 mM benzamidine, 1 µg/ml pepstatin, 4 mM EDTA). The dialyzed fraction was passed through a 0.22 µm filter (Miller-GV, Millipore Corp., Bedford, MA). The filtered solution was layered onto a mono-S (Pharmacia, New Market, NJ) column (1 cm x 10 cm, d x h) pre-equilibrated with buffer C. The column was washed with buffer C, and recombinant rPEDF was eluted with a gradient of 0 mM - 500 mM NaCl in buffer C at 0.5 ml/min. Two-ml fractions were collected, and the peak fractions of recombinant rPEDF were pooled. The recovery in the pooled fractions was 0.5 mg of recombinant PEDF per gram of packed cells.

20

EXAMPLE 6

This example describes the use of purified recombinant PEDF as a differentiation agent.

Y79 cells (ATCC, HTB18) were grown in Eagle's Minimal Essential Medium with Earl's salts (MEM) supplemented with 15% fetal bovine serum and antibiotics (10,000 u/ml penicillin and 10 mg/ml streptomycin) at 37°C in a humidified incubator under 5% CO<sub>2</sub>. Cells were propagated for two passages after receipt from the ATCC, and then frozen in the same medium containing 10% DMSO. A few of the frozen aliquots were used for each differentiation experiment. All experiments were performed in duplicate.

After thawing, the cells were kept, without further passaging, in the serum-containing medium until the appropriate number of cells were available. Cells



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° were collected by centrifugation and washed twofold in PBS, resuspended in PBS, and counted. At that point,  $2.5 \times 10^5$  cells were plated into each well of a 6-well plate (Nunc, Inc., Roskilde, Denmark) with 2 ml of serum-free medium (MEM, supplemented with 1 mM sodium pyruvate, 10 mM HEPES, 1X non-essential amino acids, 1 mM L-glutamine, 0.1% ITS mix (5  $\mu$ g/ml insulin, 5  $\mu$ g/ml transferrin, 5 ng/ml selenium, Collaborative Research, Bedford, MA), and antibiotics as described above.

10 Differentiation effectors and control buffers were added 12-16 hours after plating, and the cultures were incubated and left undisturbed for 7 days. On the eighth day, cells were transferred to poly-D-lysine-coated six-well plates (Collaborative Research, Bedford, MA), and the old medium was replaced with 2 ml of fresh serum-free medium, upon attachment of the cells to the substrate. 15 The cultures were maintained under these conditions for up to 11 days. Post-attachment cultures were examined daily for morphological evidence of differentiation as well as quantification of neurite outgrowth using an Olympus CK2 20 phase-contrast microscope.

In comparison with untreated cells, only Y79 cultures that were exposed to recombinant rPEDF showed any significant evidence of neuronal differentiation. Some neurite outgrowth (below 5%) was detectable in control 25 cultures treated with the same buffer used to solubilize rPEDF, and no evidence of differentiation was found in cultures processed in the same manner without the addition of rPEDF or buffer (Figure 22A, "control"). Phase contrast microscopy of rPEDF treated cultures showed that 30 between 50-65% of the cell aggregates had neurite extensions by day 3 post-attachment on poly-D-lysine (Figure 22B, "PEDF"). These 3-day neurite extensions appeared as short projections from pear-shaped cells at the edges of the cell aggregates. The number of 35 differentiating aggregates, the number of differentiating

- 32 -

cells per aggregate, and the length of the neurite-like processes increased with post-attachment time. By day 5 post-attachment, about 75-85% of the aggregates showed signs of differentiation with neurites extending from most of their peripheral cells. rPEDF-treated cultures reached the maximum extent of differentiation on day 7 post-attachment, when 85-95% of the cells aggregate. At that time, two types of neuronal processes were observed, i.e., single neurites 2-3 fold longer than those observed on day 3 extending from peripheral cells of isolated aggregates, and much longer and thinner processes forming a branching network between neighbor cell aggregates. Upon extended incubation, i.e., beyond 10 days post-attachment, there was a marked decrease in the proportion of the network connections, and no further growth of the single neurites, although the viability of the cell aggregates was not severely affected, and remained at about 75-80% in different experiments. No differences were observed between purified native PEDF and recombinant PEDF (rPEDF) as seen in Figure 23.

The PEDF and rPEDF cDNA clones not only provide means to produce large quantities of the PEDF and rPEDF proteins but also serve as sources for probes that can be used to study the expression and regulation of the PEDF gene. In addition, these sequences can be used in the antisense technique of translation arrest to inhibit the translation of endogenous PEDF.

The recombinantly produced PEDF and rPEDF proteins and equivalent proteins can be used as potent neurotrophic agents in vitro and in vivo. Additional biochemical activities of these proteins as neurotrophic agents can be determined through standard in vitro tests, which will enable the development of other therapeutic uses for these proteins in the treatment of inflammatory, vascular, degenerative and dystrophic diseases of the retina. Given that these proteins are such potent

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neurotrophic agents, it can be envisioned that these proteins could be modified for therapeutic utility in the treatment of tissues other than the retina, which also respond to neurotrophic factors. These proteins may even find more generic utility as "differentiation" factors for non-neural tissues and certain types of cancer.

#### EXAMPLE 7

Along with the 3,000 mol. wt. recombinant PEDF, smaller recombinant constructs have been synthesized to determine if they have neurotrophic activity. Smaller peptides could offer a variety of advantages over the full-length construct such as greater solubility, better membrane penetration, less antigenicity, greater ease in preparation, etc.

Figure 23 shows only three of the constructs that have been tested. BP, BX and BA are about 28,000, 24,000 and 9,000 mol. wts. respectively and represent C-terminal deletion mutants. All of these show neurotrophic activity similar to that depicted in Figures 21 and 22. The novel finding here is that even the 9,000 m.w. peptide (only about 20% of the full m.w. of the native protein) exhibits striking neurotrophic activity. Moreover, the active neurotrophic peptide represents sequences at the N-terminal rather than at the C-terminal which is known to contain the serpin active site. Thus, that the active site is at the N-terminal and activity can be elicited with such a small molecule are surprising findings that could not have been predicted based on any previous findings.

30

35

### TABLE 1

### Exon and Intron Organization of the human PEDF Gene

[illegible]

Table 1: Exons are in upper case and introns sequences in lower case. The 5' donor GT and 3' acceptor AG are underlined. Exon and intron sizes are given in bp and kb respectively.

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EXAMPLE 8Cloning and sequencing of the human PEDF gene.

Materials- Restriction enzymes, SuperScript® RT and Kanamycin were purchased from GIBCO-BRL (Gaithersburg, MD). Dynabeads® Oligo dT<sub>(25)</sub> were purchased from Dynal Inc. (Lake Success, NY). Retrotherm™ RT was obtained from Epicentre Technologies (Madison, WI). RNasin® was purchased from Promega (Madison, WI). Taq polymerase was purchased from Perkin-Elmer (Norwalk, CT), or Stratagene (La Jolla, CA). The plasmid vector pBlueScript® used for subcloning was purchased from Stratagene (La Jolla, CA). Total RNA from neural retina and retinal pigment epithelium was purified from human tissue obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA) as previously described (Chomczynski and Sacchi, 1987). [<sup>32</sup>P]α -dATP and [<sup>32</sup>P]γ-ATP (3000 Ci/mmol) used for labeling and sequencing (respectively) were purchased from Amersham (Arlington Hts, IL). Superbroth (Bacto-Tryptone 12g/L, yeast extract 24 g/L, K<sub>2</sub> HPO<sub>4</sub> 12.5 g/L, HK<sub>2</sub>PO<sub>4</sub> 3.8 g/L and glycerol 5 mL/L), denaturing solution (0.2 N NaOH, 1.5 M NaCl), neutralizing solution (1 M Tris-Cl pH 7.0, 1.5 M NaCl), 20X SSC (3.0 M NaCl, 0.3 mM sodium citrate), 10X TBE (1 M Tris-borate, 2 mM EDTA, pH 8.3), and 50X TAE (2 M Tris-acetate 50 mM EDTA, pH 8.0) were purchased from Quality Biologicals (Gaithersburg, MD). 20X SSPE (3M NaCl, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 20 mM EDTA pH 7.4) was purchased from Digene Diagnostics, Inc. (Silver Spring, MD). Ampicillin was purchased from Sigma Chemical Co. (St. Louis, MO) dissolved in water and filter-sterilized.

Polymerase chain reaction (PCR). A 2X PCR mix was prepared containing 1.6 μmoles/mL of GeneAmp® dNTPs (400 μM each), 2X GeneAmp® PCR buffer and 50 U/mL Taq polymerase. These reagents were purchased from Perkin-Elmer (Norwalk, CT). In general, the template and

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oligonucleotides (100 ng of each oligo) were mixed in 25  $\mu$ L volume and 25  $\mu$ L of the 2X mix were then added followed by 50  $\mu$ L of mineral oil. The template was initially denatured for 2 min at 95°C, 30 sec annealing (temperature between 55 and 65°C depending on the primers) and an extension at 72°C for 1-5 min depending on the length of the product amplified.

*cDNA synthesis on Dynabeads® oligo (dT)<sub>25</sub>*. The cDNA was synthesized on Dynabeads as previously described (Rodriguez and Chader 1992). The Dynabeads (0.5 mg) were washed with 100  $\mu$ L of 10 mM Tris-Cl pH 7.0, 1 mM EDTA, 1 M KCl. The total RNA 30  $\mu$ L, (30  $\mu$ g, ~1  $\mu$ L), in water was mixed with 30  $\mu$ L of the above buffer and the equilibrated Dynabeads (0.5 mg) then heated to 55°C for 2 minutes. The poly+ A RNA was allowed to anneal to the beads for 15 min at room temperature and the excess RNA removed by binding the beads for 15 min at room temperature and the excess RNA removed by binding the beads to the MPC-E magnetic separator (Dynal Inc.). The beads with the annealed poly+ A mRNA were then suspended in 2.5  $\mu$ L buffer A (200 mM Tris-Cl pH 8.3, 1.0 M KCl), 2.5  $\mu$ L buffer B (30 mM MgCl<sub>2</sub>, 15 mM MnCl), 20  $\mu$ L 10 mM dNTP's (2.5 mM each), 1  $\mu$ L RNasin, 2  $\mu$ L SuperScript RT, 5  $\mu$ L of Retrotherm RT (1 Unit/ $\mu$ I) and 16  $\mu$ L of H<sub>2</sub>O to make a final volume of 50  $\mu$ L. The reaction mixture was incubated at 40°C for 10 min, then at 65°C for 1 hr. The beads were again bound to the MPC-E magnetic separator and the excess RT reaction mix removed. The beads were then washed once with 100  $\mu$ L 0.2N NaOH, once with 10X SSPE, and twice in 1X TE. The cDNA-containing beads were suspended in a final volume of 100  $\mu$ L 1X TE.

*5' Rapid Amplification of cDNA Ends (RACE)*. The 5'-RACE was performed using a modified method based on the 5'-AmpliFINDER RACE kit purchased from Clontech (Rodriguez et al. 1994). First, cDNA was synthesized on Dynabeads® Oligo dT<sub>25</sub>, as described above (Rodriguez and Chader,

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1992). The AmpliFINDER anchor primer (Clontech) was ligated to the 3' ends tips of the Dynabead-immobilized retinal pigment epithelium cDNA using the same conditions as for soluble cDNA described in the 5'-AmpliFINDER RACE kit. The Ampli-FINDER anchor primer was used in combination with an PEDF-specific primer #2744 to PCR amplify the 5' prime end. The amplification was done as described above with 2  $\mu$ L of anchor-ligated human retinal pigment epithelium-Dynabeads cDNA used as template. The amplification was performed for 30 cycles.

*Sequence of oligonucleotides.* Oligonucleotide primers were synthesized in an Applied Biosystems Inc. (Foster City, CA) DNA synthesizer model 392. The oligonucleotides were deprotected and used without further purification.

*Screening of genomic libraries.* The human genomic cosmid library (Clontech) was plated on LB plates containing 150 mg/mL ampicillin, 20 mg/mL Kanamycin at a density of 10,000 colonies per plate. Nitrocellulose filters were used to lift the colonies and the filters were treated and hybridized as described in Sambrook et al., (1989). The library was probed with [ $^{32}$ P]-labeled PCR product obtained from amplifying a PEDF cDNA clone (Steele et al. 1993) using T7/T3 primers. This resulted in the isolation of the p10A cosmid. A  $\lambda$ DASH<sup>TM</sup>II library (Stratagene) was screened by Lark Sequencing Technologies Inc. (Houston, TX) using the insert from the PEDF cDNA clone mentioned above. This resulted in the isolation of the 7 Kb NotI-NotI fragment (JT6A). A P-1 clone, p147, containing the entire PEDF gene and flanking regions was isolated using oligos 1590/1591 by Genome Systems (St. Louis, MO).

*Cloning of PCR products:* Four sets of primers, 603:604; 605:606; 2238:354 and 2213:2744 designed from the internal coding regions of the PEDF cDNA sequenced were synthesized

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as described above for use as primers in a polymerase chain reaction (PCR) experiments. The primer sequences are as follows: 603: 5'-ACA AGC TGG CAG CGG CTG TC-3' (SEQ ID NO: 13), 604: 5'-CAG AGG TGC CAC AAA GCT GG-3' (SEQ ID NO: 14); 605: 5'-CCA GCT TTG TGG CAC CTC TG-3' (SEQ ID NO: 15), 606: 5'-CAT CAT GGG GAC CCT CAC GG-3' (SEQ ID NO: 16), 2213: 5'-AGG ATG CAG GCC CTG GTG CT-3' (SEQ ID NO: 17), 2744: 5'-CCT CCT CCA CCA GCG CCC CT-3' (SEQ ID NO: 18); 2238: 5'-ATG ATG TCG GAC CCT AAG GCT GTT-3' (SEQ ID NO: 19), 354: 5'-TGG GGA CAG TGA GGA CCG CC-3' (SEQ ID NO: 20). The amplifications, subcloning and sequencing of the PCR products generated with primers 603:604 and 605:606 was performed by Lark Sequencing Technologies Inc. using human genomic DNA as template. The product generated from 603:604 is ~2 kb (jt8A) and expands from exon 3 to exon 5. The product generated using 605:606 is ~3.3 kb (jt 9) and expands from exon 5 to exon 6. The primers set 2213-2744 was used to amplify a ~ 2.5 Kb product (jt15; also referred to as JT115) from the P1 clone p147. This product was then sent to Lark Sequencing Technologies Inc. for subcloning and sequencing. The 2238:354 primers were used to amplify from exon 6 to exon 7 across intron E. This product was not subcloned but was sequenced directly and entirely by us.

*DNA sequencing.* The P-1 clone (p147), subclones of this clone and PCR products from this clone were sequenced. Most of the sequencing was performed by Lark Sequencing Technologies Inc. using standard sequencing techniques. All important areas (e.g. intron-exon boundaries), and junctions between clones were sequenced in our laboratory. DNA from the PCR products was prepared for sequencing using Wizard™ PCR Preps DNA purification kit purchased from Promega Corp. (Madison, WI). The P-1 clone, and plasmid subclones were purified using Qiagen Inc. (Chatsworth, CA) Midi plasmid purification kit. The purified PCR products and plasmids were sequenced using



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the PRISM™ DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems a Division of Perkin-Elmer Corp., Foster City, CA), following the manufacturer's protocol. Typically, 0.5 pmoles of template and 3 pmoles of primer were used per sequencing reaction. The sequencing reaction products were purified using Select-D G-50 columns (5 Prime-3 Prime; Boulder, CO) and dried. Each sample was then dissolved in 5  $\mu$ L formamide, 1  $\mu$ L 50 mM EDTA, heated and loaded in a Model 370A Automated Fluorescent Sequencer (ABI, Foster City, CA). All splice-sites junctions, intron F and junctions across clones were sequenced.

*Southern blot.* An EcoRI digested genomic (8  $\mu$ g) blot of DNA from a variety of species was purchased from BIOS Laboratories, New Haven, CT. The blot was probed with the PEDF cDNA using standard techniques (Sambrook et al., 1989).

*5' RACE of PEDF.* The 5' RACE was performed as described above by ligating the anchor oligo to human retinal pigment epithelium cDNA previously synthesized on Dynabeads. The 5' end was amplified using the anchor primer (AmpliFinder's kit) and the PEDF-specific primer 2744. The amplification was performed for 30 cycles. One main band was observed at ~ 230 bp. The PCR products were cloned in pGEM-T (Promega Corp., Madison, WI) and sequenced. The longest of these clones was found to extend the 5' end of PEDF by 20 bp.

*Isolation of the PEDF gene.* The PEDF gene was isolated in a P-1 clone (p147) by Genome Systems (St. Louis, MO) using primers 1590 and 1591 (1590: 5'-GGA CGC TGG ATT AGA AGG CAG CAA A-3' (SEQ ID NO: 23); and 1591: 5'-CCA CAC CCA GCC TAG TCC C-3' (SEQ ID NO: 24)). In order to determine if this clone contained the entire PEDF gene, both p147 and human genomic DNA were digested with BamHI, EcoHI, HindIII and PstI then separated by agarose

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gel electrophoresis in a pulse field apparatus. The agarose gel was blotted and probed with the PEDF cDNA clone (Steele et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:1526-1530). Comparison of the band pattern between the P-1 clone and genomic DNA indicates that the entire PEDF gene is contained in this clone. Furthermore, this result is also indicative that there is only one gene for PEDF.

*Sequence of the PEDF gene.* A scale map of the gene is shown in Fig. 1. The PEDF gene was sequence in its entirety (SEQ ID NO:43). The clones jt1, jt14, jt6A and related PCR products (jt15, jt8A and jt9) (Fig. 1) were sequenced by Lark Sequencing Technologies Inc. The rest of the gene was sequenced by amplifying different portions of the gene using the p147 clone as template. All exons, intron-exon junctions and the entire intron F were sequenced in both directions in our laboratory as described above from PCR products generated from the P-1 clone, p147. The Not I site downstream from exon 1 was also confirmed by amplifying across it and sequencing the product. The gene expands approximately 16 Kb with 8 exons. All intron-exon junctions obey the AG/GT rule. The intron-exon junctions and flanking sequences are shown in Table I.

jt1: A 7.1 kb cosmid clone isolated from a human genomic cosmid library (Clontech) containing exon 7, exon 8 and the 3' flanking region of the PEDF gene. The 5' end of this clone, an area of approximately 2.1 Kb, is not part of PEDF. This was apparently caused by a rearrangement of the cosmid. This clone was sequenced entirely by Lark Sequencing Technologies Inc.

jt6A: This is a 7.2 kb Not I fragment isolated by Lark Sequencing Technologies Inc. from a  $\lambda$ DASHII human genomic library (Statagene). This clone contained >6 Kb of the 5' flanking region, exon1 and 424 bp of intron A of the PEDF

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- gene. This clone was sequenced entirely by Lark Sequencing Technologies Inc.

jt8A: This cloned PCR product JT8A generated from genomic DNA using primers 603:604. This clones expands from exon 3 to exon 5 including exon 4 and introns C and D. It was amplified, cloned and sequenced entirely by Lark Sequencing Technologies Inc.

jt9: This cloned PCR product JT8A was generated from genomic DNA using primers 605:606. It contains the entire intron E and portions of exon 5 and exon 6. It was amplified, cloned and sequenced entirely by Lark Sequencing Technologies Inc.

jt15: This clone was obtained from a PCR product amplified using the primer pair 2213:2744 from p147. The clone expands from exon 2 to exon 3 across intron B. The PCR product was submitted to Lark Sequencing Technologies Inc. for subcloning and sequencing.

P1 clone p147: This clone was isolated by Genome Systems Inc. using oligonucleotides 1590:1591. This clone was used to obtain the sequence of intron F (2238:354), and the subclone jt14. It was also used to confirm the intron-exon boundaries initially obtained from the above mentioned clones. All the exons and intron boundaries were amplified (using p147 as template) using intron-specific oligos and the products sequenced. All splice junctions sequences were confirmed as well as the sizes of introns and exons.

jt14: This is a subclone of p147 containing most of intron A, exon 2 and a portion of intron B. This clone was isolated by us and sent to Lark Sequencing Technologies Inc. for sequencing.

Thus from the sequence analysis of all the above mentioned clones and PCR products the structure and size of exons and introns of the human PEDF gene were determined. The 5' splice donor and 3' splice acceptor sites in all junctions conform to the GT/AG consensus.

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EXAMPLE 9Analysis of the PEDF promoter.

In order to obtain some understanding as to the possible transcriptional elements that may regulating PEDF and guidance for future experiments on PEDF expression, we performed a theoretical analysis of the PEDF 5' flanking region (Fig. 3). The 5' flanking region of the PEDF gene lacks the classical TATAAA signal or TATA-box. However, it contains several interesting features and elements recognized by important transcription factors. There are two Alu repetitive elements from -164 to -591, and from -822 to -1050. Outside the Alu regions, there are two possible sites for the ubiquitous octamer family of transcription factors (Oct) at -29 (ATCCAAAT) and again at -113 (GTGCAAAT) which deviate by one base from the consensus ATGCAAAT (Parslow et al. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81:2650-2654; Falkner et al. (1984) *Nature* 310:71-74; Sturm et al. (1988) *Genes & Devel.* 2:1582-1599; Faisst and Meyer (1992) *Nuc. Acids Res.* 20:3-26). Another element of possible interest is located at -62. This element, GTAAAGTTAAC, which resembles the HNF-1 (hepatocyte nuclear factor) binding consensus GTAATNATTAAC (Frain, M., et al. (1989) *Cell* 59:145-147). This is a homedomain-containing transcription factor which transactivates many predominately hepatic genes (Kuo et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:9838-9842) but has been implicated in endodermic differentiation (Baumhueter et al. (1990) *Genes Dev.* 4:371-379). The sequence TCAGGTGATGCACCTGC at -202 is very similar to the artificial palindromic sequence (TREp) TCAGGTCATGACCTGA which is recognized by AP-1 and possibly transactivated by retinoic acid (Umescono et al. (1988) *Nature* 336:262-265; Linney (1992) *Curr. Topics in Dev. Biol.* 27:309-350). The sequences TGAGTGCA at -22 and TGATGCA at -207 (within the TREp), are similar to the AP-1 consensus sequence TGA

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° (Schüle, et al. (1990) *Cell* 61:497-504). The sequence AGGTGATGCACCT at -204 contained within the TREp is also similar to the developmentally regulated RAR (retinoic acid receptor) motif whose consensus is AGGTCATGACCT (Faisst and Meyer (1992) *Nuc. Acids Res.* 20:3-26). The  
5 PEA3 element (polyomavirus enhancer activator 3) AGGAAG/A (Martin et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5839-5843; Faisst and Meyer (1992) *Nuc. Acids Res.* 20:3-26) is present in tandem at -122 and -129, then again at -141. PEA3 is a member of the ETS family of transcription  
10 factors (Macleod et al. (1992) *TIBS* 17:251-256) and its activity seems to be regulated by non-nuclear oncogenes (Wasylyk et al. (1989) *EMBO J.* 8:3371-3378). One of the most interesting elements is located at -654 with the sequence GTGGTTATG. This element is within the consensus  
15 sequence GTGGT/AT/AT/AG recognized by the C/EBP (CAAT enhancer binding protein) family of transcription factors (Faisst and Meyer (1992) *Nuc. Acids Res.* 20:3-26). This factor seems to be involved in terminal differentiation that leads to an adult phenotype (Vellanoeweth et al.  
20 (1994) *Laboratory Investigation* 70:784-799). Three possible CACCC boxes are present one at -845 and two in the reverse orientation at -826 and -905. These are all within the Alu repeat. A possible Sp1 site (CCCGGC) is present at -153 before the Alu repeat and a consensus Sp1  
25 site GGCGGG is present -1030 inside the Alu repeat.

#### EXAMPLE 10

##### Expression of PEDF mRNA in Cultured Cells

##### Gene expression analysis

30 Multiple human tissue mRNA Northern blots (Clontech) with 2 ug Poly-(A) RNA per lane were hybridize with a radioactively-labelled 667 bp PCR amplified PEDF product (Tombran-Tink et al., 1994 Genomics, 19:266-272). Blots were prehybridized for 15 min at 68°C in QuickHyb  
35 rapid hybridization solution (Stratagene, La Jolla, CA)

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and hybridized for 1 hr at 68°C in the same solution containing  $5 \times 10^6$  cpm DNA/ml. Hybridized blots were washed twice with 100 ml of 2XSSC, 0.1% SDS for 15 min at room temperature and once with 200 ml of 0.1XSSC, 0.1% SDS for 30 min at 68°C. The blots were autoradiographed at -  
5 70°C for 2 hr using Kodax XAR-5 film and DuPont intensifying screens.

#### Gene Expression:

In order to determine whether expression of the PEDF messenger RNA occurs in human tissues other than in  
10 cultured human fetal RPE cells, we analyzed multiple tissue human adult and fetal RNA blots containing equal amounts of poly-(A) RNA for each tissue examined. The results are shown in Figure 4. The PEDF probe identified  
15 a single primer 1.5 kb transcript of varying intensity of hybridization in 14 of the 16 adult tissue analyzed. No signal is detected in either adult kidney or peripheral blood leucocytes. Only a weak signal can be observed in adult brain, pancreas, spleen and thymus. The greatest  
20 amount of hybridization for PEDF messenger RNA is seen in human adult liver, skeletal muscle, testis and ovary. Surprisingly, only a very weak signal is observed in total brain RNA. In the fetal tissues examined, a very strong PEDF signal is seen in liver tissue, and interestingly a  
25 signal of significant intensity in fetal kidney as compared to no PEDF hybridization in adult kidney samples.

In contrast to the single 1.5 kb transcript observed in the adult tissues, an additional minor transcript of less than 500 bp is labelled variably and  
30 with lower intensity in fetal heart, lung and kidney. This may be due to partial degradation of the message or an alternative splicing phenomenon. PEDF is also only expressed in early passaged monkey RPE cells (1st - 5th passage) and not in late passaged cells (10th passage).

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These data demonstrate the relevance of PEDF to senescence.

EXAMPLE 11

Comparative Analysis Of PEDF In  
A Variety Of Phylogenetically Related Species

Evolutionary conservation analysis

8 ug of genomic DNA from lymphocytes of a variety of species including a number of mammalian and primate species (BIOS laboratories, New Haven CT.) was digested with Eco-R1 and separated in 1% agarose gels. The gels were transblotted and membranes containing the digested DNA hybridized using the same procedure and conditions as that for Northern analysis.

Evolutionary conservation:

The evolutionary conservation of PEDF among a number of phylogenetically related species was examined. The results are presented in Figure 5. Using these high stringency hybridization conditions, a large EcoRI restriction fragment of approximately 23 kb is observed in aves, mammals and primates. No hybridization signals were seen in lower species (Figure 5A) possible due to weak homology of the human PEDF probe used. The EcoRI fragment for both chicken and mouse is somewhat smaller than that for humans. An interesting restriction pattern emerges in several of the mammalian species examined (Figure 5B). Several smaller restriction fragments ranging in size between 6 kb and 2 kb are seen. The larger fragments range in size between 9 kb and 23 kb and are seen in all primates species examined which has an additional strongly hybridizing polymorphic fragment at approximately 9 kb.

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EXAMPLE 12Neuronotrophic Effects of Pigment Epithelium  
Derived Factor On Cerebellar Granule Cells In CultureCell Culture

5 Cerebellar granule cells (CGC) were prepared  
from 5 or 8-day-old Sprague-Dawley rat pups as described  
by Novelli et al. (1988, *Brain Res.*, 451:205-212). In  
brief, tissue free of meninges was minced in a buffer  
10 containing 124 mM NaCl, 1mM NaH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 3 mg/ml  
bovine serum albumin (BSA), 27 µM phenol red, and 25 mM  
HEPES (pH 7.4), and centrifuged at 550 xg for 3 min. The  
tissue pellet from 10-20 animals was resuspended and  
trypsinized (15 min, 37°C) in 30ml of the same buffer  
15 containing 250 µg/ml trypsin; a further 15 ml of buffer  
was added containing 26 µg/ml DNase I, 166 ug/ml soybean  
trypsin inhibitor, and 0.5 mM additional MgSO<sub>4</sub> and the  
tissue was centrifuged again as described above. The  
pellet was resuspended in 1 ml of buffer supplemented with  
80 µg/ml DNase, 0.52 mg/ml of trypsin inhibitor, and 1.6  
20 mM additional MgSO<sub>4</sub>, and triturated 60 times with a  
Pasteur pipette. The suspension was diluted with 2 ml of  
buffer containing 0.1 mM CaCl<sub>2</sub> and 1.3 mM additional MgSO<sub>4</sub>,  
and undissociated material allowed to settle for 5 min.  
The supernatant was transferred to another tube, cells  
25 were recovered by brief centrifugation and resuspended in  
serum-containing medium (Eagle's basal medium with 25 mM  
KCl, 2 mM glutamine, 100 µg/ml gentamycin, and 10% heat  
inactivated fetal calf serum) or chemically defined medium  
(DMEM:F 12 (1:1) with 5 µg/ml insulin, 30 nM selenium, 100  
30 µg/ml transferrin, 1000 nM putrescine, 20 nM progesterone,  
50 U/ml penicillin, 50 µg/ml streptomycin, and 2 mM  
glutamine) (Bottenstein, 1985 Cell Culture in the  
Neurosciences, J.E. Bottenstein and G. Sato, eds. New York  
Plenum Publishing Corp. p. 3-43). Cells were plated in  
35 poly-L-lysine-coated 96 well plates (for MTS assay and



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- ° neurofilament ELISA assay) or 8-well chamber slides (for immunocytochemistry and BrdU labelling) at  $2.5 \times 10^5$  cells/cm<sup>2</sup> and grown at 37°C in an atmosphere consisting of 5% CO<sub>2</sub> in air. After 1 day in culture, cytosine arabinoside (Ara-C) was added only to cells in serum-supplemented medium (final concentration 50 µM).

#### MTS Assay

- Cerebellar granule cells in 96 well plates were incubated in a CO<sub>2</sub> incubator for 4 hours with MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and PMS (phenazine methosulfate) final concentration; 333 µg/ml MTS and 25 µM PMS (Promega Corp.). In the presence of PMS, MTS is converted to a water-soluble formazan by a dehydrogenase enzyme found in metabolically active cells (Cory et al. (1991) *Cancer Comm*, 3:207-212). The quantity of formazan product was determined by spectrophotometry at 490 nm.

#### Immunocytochemistry

- After 7 days *in vitro* (DIV), the cells were washed three times in calcium-and magnesium-free phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde for 10 min, followed by 10 min at -20°C in 95% ethanol/5% acetic acid. Incubation with primary antibodies against NSE (neuron specific enolase), GABA, calbindin, or glial fibrillary acidic protein (GFAP) was carried out for 60 min at RT. Antibodies were applied at 1:1000-1:5000 in the presence of 2% normal goat serum and 0.2% BSA. The antibodies were visualized using the ABC system (Vector Laboratories) and diaminobenzidine. At least 20 fields were counted from 2-3 wells for each experiment. The average number of cells per field was then calculated to determine the ratio for the number of cells stained by the other antibodies relative to NSE-positive cells in control cultures.

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### Bromodeoxyridine (BrdU) Labeling

BrdU labeling was performed by the method of Gao et al. (1991 *Neuron*, 6: 705-715) with the following modification. The cells were plated in 8-well chamber slides and rPEDF added immediately. After 24 hours, BrdU (1:100; Amersham cell proliferation kit) was added to the culture medium for 24 hours, after which the cells were fixed in 2% paraformaldehyde (10 min), treated with 95% ethanol / 5 acetic acid (10 min), and incubated with an anti-BrdU monoclonal antibody (1:20 for 2 hrs). The cultures were then incubated with a horseradish peroxidase-conjugated goat anti-mouse secondary antibody for 60 min. After diaminobenzidine-peroxidase, the cells were mounted in Gel Mount. The mitotic index was determined by counting the percentage of labeled cells with a microscopy. For each value, a random sample of 3000 cells was counted.

### Neurofilament ELISA Assay

The neurofilament ELISA was performed according to the method of Doherty et al. (1984 *J. Neurochem.*, 42:1116-1122) with slight modification. Cultures grown in 96-well microtiter plates were fixed with 4% paraformaldehyde in PBS at 4°C for 2 hr. The fixed cells were permeabilized by treatment for 15 min with 0.1% Triton X-100 in PBS, followed by incubation for 60 min with PBS containing 10% goat serum to block nonspecific binding. The cultures were then incubated with a monoclonal anti-neurofilament antibody overnight at 4°C (RMO-42 at 1:100; which stains only neurites in the cultures of cerebellar granule cells). After washing twice with PBS containing 10% goat serum, cells were incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse at 1:1000) for 1 hr. Following sequential washing with PBS and water, the cultures were incubated with 0.2% O-phenylenediamine and 0.02% H<sub>2</sub>O<sub>2</sub> in 50

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° mM citrate buffer (pH 5.0) for 30 min. The reaction was stopped by adding an equal volume of 4.5 M H<sub>2</sub>SO<sub>4</sub>. Product formation was quantitated by reading the optical density (O.D.) of an aliquot of the reaction product at 490 nm using a microplate reader.

5 In order to validate the MTS assay as a measure of live cells, and to determine the range of cell number over which the results would be linear, the experiments shown in Figure 6 were carried out. In serum-containing medium (SCM) (Figure 6A), optical density (O.D.) was  
10 proportional to cell number plated over a range from  $1-9 \times 10^5$  cells/cm<sub>2</sub>. In contrast, for cells grown in chemically-defined medium (CDM) (Figure 6B), the linear range covered  $1-5 \times 10^5$  cells/cm<sup>2</sup>. For all subsequent experiments, cells  
15 were plated at  $2.5 \times 10^5$  cells/cm<sup>2</sup>, in the middle of the linear range for either type of culture medium.

Figure 7 shows that PEDF caused a significant increase in cell number by DIV4 with a larger difference at DIV7 and 10. However, the 2-3 fold increases were the  
20 result of large decreases in cell numbers in the control cultures. The dose-response curve in chemically-defined medium (Figure 8), showed that there is a statistically significant effect at 20ng/ml. Increasing the concentration of PEDF above 50 ng/ml did not produce  
25 further increases in CDM.

30 In order to determine whether the increase in O.D. (MTS assay) in response to PEDF reflected an increase in surviving cells or an increase in proliferation, a BrdU labeling study was performed using cultures from postnatal day 5 (P5) animals (a time when cerebellar granule cells are still dividing in the animal). Figure 9 shows the effect of PEDF on P5 CGC cultures at DIV1 and 2. Using the MTS assay, PEDF had no effect at DIV1 but caused a small increase in O.D. at DIV2 in either serum-containing medium or chemically defined medium. Therefore, BrdU was  
35

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° added at day 1 and cells were fixed on day 2. The BrdU labeling index was 5% in SCM and 3% in CDM, under control conditions, and PEDF did not increase the BrdU labeling index in either culture medium (Figure 10). The lack of stimulation of the BrdU labeling index by PEDF implies  
5 that enhanced survival rather than increased cell division is responsible for the increased O.D. measured by the MTS assay after exposure to PEDF.

Immunocytochemistry was used to identify the cells present in cultures before and after treatment with  
10 PEDF. P8 cultures grown for 7 days with and without PEDF (500 ng/ml) were stained with four different antibodies: a polyclonal rabbit antibody to neuron-specific enolase (NSE), which recognizes all cerebellar neurons (Schmechel et al. (1978) *Science*, 199:313-315); a polyclonal antibody  
15 to GABA, which is synthesized in all cerebellar neurons except cerebellar granule cells (Gruol and Crimi (1988) *Dev. Brain Res.*, 41:135-146); an antibody to calbindin, which is a neuron-specific protein and GFAP, an intermediate filament protein present only in astrocytes.  
20 The results are summarized in Table 2. PEDF significantly increased the number of NSE-positive cells in both SCM (30% increase) and in CDM (60% increase). There was a small, not statistically significant, increase in the number of GABA-positive neurons and Purkinje cells  
25 (calbindin-positive). Thus, PEDF is neurotrophic only for granule neurons. In addition, PEDF significantly decreased the number of GFAP-positive astrocytes present in the cultures (30% decrease in SCM and 40% decrease in CDM). This "gliastatic" property of PEDF is further  
30 discussed in Example 14.

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TABLE 2

Immunocytochemistry demonstrates that PEDF Increased The Number of NSE-Positive Cells (Neurons) But Decreased GFAP-Positive Cells (Glia)

Antigen	Treatment	SCM	CDM
NSE	Control	100.0 $\pm$ 6.2	100.0 $\pm$ 4.5
	PEDF	127.0 $\pm$ 5.9*	157.2 $\pm$ 7.4*
GABA	Control	2.8 $\pm$ 0.2	1.4 $\pm$ 0.2
	PEDF	3.2 $\pm$ 0.2	1.8 $\pm$ 0.2
Calbindin	Control	0.06 $\pm$ 0.01	0.07 $\pm$ 0.02
	PEDF	0.07 $\pm$ 0.02	0.12 $\pm$ 0.02
GFAP	Control	0.86 $\pm$ 0.07	0.99 $\pm$ 0.07
	PEDF	0.60 $\pm$ 0.03*	0.60 $\pm$ 0.06*

Postnatal-day 8 cerebellar granule cells were cultured in 8-well chamber slides. PEDF (500 ng/ml) was added at DIV 0, the cells were fixed on DIV 7, and the immunocytochemistry was carried out using antibodies against NSE, GABA, Calbindin and GFAP. At least 20 fields were counted from 2-3 wells for each experiment. Data are expressed as percent of control of NSE-positive cells. Each experiment value represents mean cell number  $\pm$  SEM. \*P<0.005 compared with each other control by using non-paired test.

In order to investigate the effects of PEDF on neurite outgrowth, a neurofilament ELISA assay was used. Immunocytochemistry had shown that the monoclonal antibody RMO-42, stained only the neurites of cerebellar granule cells in culture, so this antibody was used as a direct measure of neurofilament present only in processes and not the cell body (Figure 11). PEDF slightly increased neurofilament content, both in SCM and CDM, but the increase was directly proportional to the increase in cell number (Figure 12).

Figure 13 summarizes the data from this Example. By 10 days in culture, most untreated CGCs die (control) but 60% or more of the PEDF-treated cells remain viable. PEDF is thus a potent survival factor for brain neurons.

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EXAMPLE 13

Neuronotrophic properties of rPEDF peptides, BP and BX.

Described in the previous sections on the "neuronotrophic" activity of PEDF is the fact that we can produce relatively large amounts of a recombinant PEDF (rPEDF) that exhibits potent neurotrophic activity. Using appropriate recombinant molecular biological technology, we can also produce smaller fragments of the PEDF molecule that can be tested for either neurotrophic or neuronotrophic activity. Figure 14 shows the effects of two of these truncated forms of PEDF on CGC viability. BX and BP are 24 and 28 kDa fragment from the amino-terminal portion of the PEDF molecule, respectively. Both fragments at 1x or 10x concentrations act as neuron-survival factors, significantly promoting the life of the CGC's. In this experiment, the peptide was given once at the beginning of the experiment and the cell number was determined 7 days later. We conclude that, along with the full PEDF molecule, smaller recombinant peptides near the N-terminal of the molecule are "neuronotrophic".

EXAMPLE 14

Gliastatic properties of PEDF

Along with neurons in the primary cultures of rat cerebellar granule cells are a small number of different types of glia. Glia are the "support" elements in the CNS for neurons, forming the architectural framework and the metabolic support system on which neurons depend. Glia are also of clinical importance since tumors of the brain are mostly formed by glia and gliosis is a problem in several neurodegenerative diseases. In our system, we first noticed an effect of PEDF on glia when we immunocytochemically stained the cultured mixed population of cells with antibodies specific for neurons and other antibodies specific for different types of glia. For this purpose, we used the standard markers Neuron-Specific Enolase (NSE) and others

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to demonstrate the presence of neurons, Glial Fibrillary Acidic Protein (GFAP) to demonstrate the presence of astroglia and OX-42 to stain microglia. In this experiment (Table 2), we found the expected increase in NSE staining with PEDF treatment since we then knew that the neurons were living longer but we found an unexpected decrease in GFAP staining. This indicated the possibility of fewer astrocytes in the PEDF-treated cultures.

Because of the distinctive morphology of astroglia and microglia in the culture dishes and their selective staining for GFAP or OX-42, it is possible to individually count their numbers under the microscope under different experimental conditions. This has now been done as outlined in Figures 15 and 16. Figure 15 shows the effects of PEDF on numbers of astroglia in cultures obtained from rat brain at 2 weeks (2w) or 12 weeks (12w) in culture. Times given are 48 hrs, 96 hrs or 7 days after treatment with PEDF. Clearly, under all the conditions tested, PEDF treatment results in a dramatic decrease in the number of astroglia. Figure 16 shows a parallel analysis of microglia in the same cultures. Administration of PEDF for 48 hrs. or 7 days resulted in fewer numbers of the cells whether they have been cultured for 2 weeks (2W) or 12 weeks (12W). Thus, PEDF substantially decreases glial elements over a very long period of time while acting as a survival factor for neurons.

#### EXAMPLE 15

##### Characterization of Native Bovine PEDF

Since the specific antibody indicated the presence of PEDF in the adult IPM, we used bovine IPM washes as a source for purification of native PEDF. Although RPE and retinal cells express PEDF mRNA, anti-BH could not detect PEDF bands on Western transfers in these cell extracts, suggesting a rapid PEDF release into the IPM. We now estimate that PEDF is present in bovine IPM

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° at less than 1% of the total soluble protein (i.e. about 2-5 ng/bovine eye). At physiological temperatures, the PEDF protein in the IPM remains stable for extended periods of time and does not form non-reduced complexes resistant to SDS. Thus, its potential usefulness in culture experiments and transplantation *in vivo* is greatly enhanced due to its stable nature.

Purification to apparent homogeneity is achieved by a simple two-step procedure (Figure 17). Components of IPM were fractionated by size-exclusion column chromatography (TSK-3000). The PEDF-immunoreactive fractions were pooled, applied to a cation-exchange column (Mono-S) and immunoreactivity was eluted with a NaCl linear gradient. Purification protocol is detailed in Materials and Methods. Elution profiles of each chromatography are shown in: panel A, TSK-3000 size-exclusion column chromatography, and panel B, mono-S column chromatography. Absorbance at 280 nm is represented by \_\_\_\_\_, and NaCl concentration by ---, PEDF-immunoreactivity was followed with antiserum Ab-rPEDF. The inserts correspond to Western blot analysis of the indicated fractions. Immunoreaction was performed with a 1:10,000 dilution of Ab-rPEDF and stained with 4-chloro-1-naphthol. Molecular size standards for the TSK-3000 chromatography were: BSA, bovine serum albumin (66,000); and CA, bovine carbonic anhydrase (29,000).

Starting with a wash of soluble IPM components, the first step involves removal of the most abundant protein, IRBP, by size exclusion chromatography. PEDF elutes as a monomeric polypeptide around 50 kDa in size. Since we have determined that PEDF's isoelectric point is 7.2-7.8, we have used S-sepharose column chromatography at pH 6.0 in the second step of our procedure to simultaneously purify and concentrate the protein. Purified protein is recovered at about 2 ug protein per adult bovine eye with a recovery of about 40%. Native



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PEDF behaves like a monomeric glycoprotein with an apparent molecular weight of  $49,500 \pm 1,000$  on SDS-PAGE.

The purified protein is sensitive to glycosidase F, revealing N-linked oligosaccharides that account for up to 3,000-Mr of the native protein (Figure 18). To remove asparagine-linked oligosaccharides purified PEDF protein was treated with endoglycosidase H and N-Glycosidase F. Enzymatic reactions were performed as described in Materials and Methods with a total of 200 ng of PEDF protein in the presence or absence of  $\beta$ -mercaptoethanol. Reactions mixtures were applied to SDS-12.5% polyacrylamide gel. Photographs of western transfers of endoglycosidase H (left panel) and N-Glycosidase F (right panel) reactions are shown. Immunoblots were treated with antiserum Ab-rPEDF diluted 1:10,000. Addition in each reaction are indicated at the top. The numbers at the right side of each photograph indicate the migration of biotinylated SDS-PAGE standards: bovine serum albumin (66,200), ovalbumin (45,000) and bovine carbonic anhydrase (31,000). We have shown that purified bovine PEDF promotes neurite outgrowth on Y-79 cells and Weri retinoblastoma cells, and that this activity is blocked by Anti-rPEDF (see below).

The present invention provides the tools for determining the effect of authentic PEDF on the expression of neuronal and glial markers in the CGC cultures and Y-79 tumor cells including NSE, GFAP, neurofilament (NF-200) protein.

#### EXAMPLE 16

##### Pigment Epithelium-Derived Factor: Characterization Using A Highly Specific Polyclonal Antibody

We have used purified recombinant human PEDF produced in *E. coli* to develop polyclonal antibodies against PEDF. Anti-rPEDF specifically recognized one polypeptide on Western transfer of IPM wash from adult bovine eyes (Figure 19). Polyclonal antiserum to human

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° recombinant PEDF specifically recognizes rPEDF. Western transfer and slot blot of human rPEDF were treated with rabbit polyclonal antiserum to rPEDF, Ab-rPEDF. Photographs of immunostaining with 4-chloro-naphthol are shown. Panel A, Western transfers of 0.5  $\mu$ g of rPEDF were used to assay increasing dilutions of antiserum. rPEDF protein was resolved by SDS-12.5% PAGE before transfer. Dilutions are indicated at the top of each lane. Diluted antiserum was preincubated with rPEDF at 5  $\mu$ g/ml before using for immunodetection and is indicated as 1:10,000+rPEDF. The numbers to the left indicate the molecular weight of biotinylated SDS-PAGE standards. Panel B increasing amounts of rPEDF in 1% BSA/PBS were applied to a nitrocellulose membrane with a manifold. The membranes were treated with antiserum Anti-rPEDF and rabbit preimmune serum diluted 1:10,000. The numbers to the right indicate the amounts of rPEDF protein blotted on the membrane. The sera used in each paper are indicated at the top of the figure.

Anti-BH specifically recognizes human PEDF on Western transfers at dilutions as low as 1:50,000; importantly, it does not recognize serum  $\alpha_1$ -antitrypsin. The antibody recognizes one major band on Western transfers of conditioned medium from juvenile monkey RPE cells in culture as well as of IPM from adult bovine eyes. Anti-rPEDF blocked the IPM-promoting neurotrophic activity (Figure 20). Human retinoblastoma Y-79 cells exponentially growing in serum containing medium were washed twice with PBS, and plated ( $2.5 \times 10^5$  cell per ml) in serum-free MEM supplemented with insulin, transferring and selenium (ITS mix, Collaborative Research Products). Effectors were then added to the cultures. After 7 days at 37°C in 5% CO<sub>2</sub>, the cells were attached to poly-D-lysine coated plates with fresh serum-free medium. The differentiation state of the cultures was monitored at different intervals after attachment. Morphology characteristic of 9-day

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post-attachment cultures is shown. Addition of effectors were as indicated in each panel at the following final concentrations: 125  $\mu$ g/ml BSA, 1% IPM, and 100 ng/ml purified bovine PEDF. In order to block the neurite outgrowth inducing activity each effector was preincubated with an excess of antiserum Anti-rPEDF (1  $\mu$ l) in 1% BSA/PBS at 4°C for at least 6 hours. All photographs are shown at x50 magnification.

The anti-rPEDF also blocked the neurite-outgrowth activity promoted by the purified PEDF. Our data indicate that PEDF is the only neurotrophic factor in the IPM. These results also suggest that the anti-rPEDF will be useful in probing the PEDF neurotrophic active site as well as the physiological role of PEDF in the IPM and other tissues (e.g. brain) as well. Further, these results indicate that PEDF is a bona fide component of the IPM and is probably the sole neurotrophic component in the extracellular matrix. Moreover, the protein is present in a wide range of tissues and extracellular spaces. The blocking antibody is useful in studies probing the physiological functions of PEDF.

#### EXAMPLE 17

##### Pigment Epithelium-Derived Factor: A Serpin With Neurotrophic Activity

The amino acid sequence derived from a fetal human PEDF cDNA shares identity of its primary structure (~30%) with the serine protease inhibitor (serpin) family, preserving 90% of the residues essential for the structural integrity of serpins. However, recombinant PEDF does not inhibit the serine proteases trypsin, chymotrypsin, elastase or cathepsin G. A natural target for PEDF has not yet been identified. We have analyzed proteins from the interphotoreceptor matrix (IPM), the space between the retinal pigment epithelium and the retina by immunodetection on Western blots with antibodies raised against PEDF and by zymography in gels containing

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° casein as a proteolytic substrate. Our results show that bovine IPM contains a stable, glycosylated PEDF polypeptide (50,000 Mr) at about 2-5 $\mu$ g per eye. Limited proteolysis of bovine PEDF produced a polypeptide of 46,000 Mr with trypsin, subtilisin, chymotrypsin and elastase, suggesting a globular structure with a hinge region susceptible to proteolytic cleavage. On the other hand, casein SDS-PAGE zymography revealed low protease activity in the IPM which migrated as a double of about 80,000  $\pm$  5,000 Mr. The caseinolytic activities were inhibited 100% with 1  $\mu$ g/ml aprotinin and 10mM PMSF added to the gel mixture, but were not affected by E64 or EDTA. Importantly, IPM protein did not react with antibody against plasminogen, a serine protease of about 80,000 Mr. When rPEDF protein was added at 1  $\mu$ g/ml, the signal for these caseinolytic activities, as well as another serine protease activity of unknown origin, diminished by about 50%. Our results suggest the IPM as a natural extracellular site for a novel serine protease and the serpin PEDF, both present at  $\leq$ 1% of the total protein.

20 All of the references cited herein are hereby incorporated in their entirety by reference.

The present invention discloses the general structural features of PEDF and beginnings of understanding of how these relate to function of the protein. PEDF possesses the structural features and general tertiary characteristics previously attributed to serpins but not its anti-protease activity. PEDF is a neurotrophic protein and appears to be the sole component of the IPM that promotes neurite-outgrowth on retinoblastoma cells. However, the reactive center for serine protease inhibition found near the carboxy terminal of classical serpins is not necessary for PEDF's neurotrophic biological activity. Specifically, a polypeptide chain containing a domain from the amino-terminal portion of the molecule (BA) is sufficient for

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° neurotrophic and neuron-survival activity. The present invention further allows for determination of whether the CGC neurons normally die by apoptosis and whether PEDF is an apoptosis inhibitor. In other words, the present invention allows one to determine by what mechanism PEDF  
5 "saves" neurons and "inhibits" glia growth or proliferation.

The present invention is useful in determining the specific neurotrophic "active site". Further, the use of rPEDF truncated peptides allows us to define the  
10 elements necessary for neuronotrophic and perhaps gliastatic activity of PEDF. The present invention further provides necessary tools to study the interactions of PEDF that trigger the signal for differentiation of retinoblastoma. Recent experiments demonstrate that <sup>125</sup>I-  
15 BH binds to retinoblastoma cells in competitive fashion only when added in medium that had been previously "conditioned" by retinoblastoma cells. This suggests that one or more co-factors produced by the cells could be required for binding. The present invention further  
20 provides the tools necessary to identify and characterize a putative cell-surface receptor for PEDF or for a PEDF complex from our CGC and retinoblastoma test systems.

Recombinant mutated proteins, proteolytic products and synthetic peptides have become instrumental  
25 in domain mapping of functional sites of proteins. Further, the recombinant proteins of the present invention allow the mapping of neurotrophic and neuronotrophic "active sites" on the PEDF molecule and the determination of the cellular transduction mechanism through which this  
30 interesting protein exerts its dramatic biological effects.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the  
35 preferred nucleic acids coding for, and the amino acid

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° sequences of, PEDF, rPEDF, and equivalent proteins, (BP, BX, BA) the vectors utilizing any such nucleic acids, the recombinant methods of producing such proteins, and the methods of using such proteins, may be realized and that it is intended that the invention may be practiced  
5 otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

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## SEQUENCE LIST

## (1) GENERAL INFORMATION:

- 5 (i) APPLICANTS: Chader, Gerald J.; Becerra, Sofia  
Patricia; Schwartz, Joan P.;  
Taniwaki, Takayuki
- (ii) TITLE OF INVENTION: PIGMENT EPITHELIUM  
DERIVED FACTOR: CHARACTERIZATION GENOMIC  
ORGANIZATION AND SEQUENCE OF THE PEDF GENE
- 10 (iii) NUMBER OF SEQUENCES: 43
- (iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Morgan & Finnegan, L.L.P.  
(B) STREET: 345 Park Avenue  
(C) CITY: New York  
(D) STATE: New York  
(E) COUNTRY: USA  
(F) ZIP: 10154
- 15 (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: Floppy Disk  
(B) COMPUTER: IBM PC Compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: WORDPERFECT 5.1
- 20 (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NO: TO BE ASSIGNED  
(B) FILING DATE: 06-JUN-1995  
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NO: 08/367,841  
(B) FILING DATE: 30-DEC-1994
- 25 (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/257,963  
(B) FILING DATE: 07-JUN-1994
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 07/952,796  
(B) FILING DATE: 24-SEP-1992
- 30 (viii) ATTORNEY/AGENT INFORMATION:  
(A) NAME: DOROTHY R. AUTH  
(B) REGISTRATION NUMBER: 36434  
(C) REFERENCE/DOCKET NUMBER: 20264126PCT
- 35 (ix) TELECOMMUNICATION INFORMATION:  
(A) TELEPHONE: (212) 758-4800  
(B) TELEFAX: (212) 751-6849

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## ° (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1512 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

5

## (ii) MOLECULE TYPE: cDNA to mRNA

## (ix) FEATURE:

- (A) NAME/KEY:
- (B) LOCATION:
- (D) OTHER INFORMATION: PEDF coding region

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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	ATCCACAGGC CCCAGGATGC AGGCCCTGGT GCTACTCCTC	160
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	CTGCCAGCCC CCCGGAGGAG GGCTCCCCAG ACCCCGACAG	240
20	CACAGGGGCG CTGGTGGAGG AGGAGGATCC TTTCTTCAA	280
	GTCCCCGTGA ACAAGCTGGC AGCGGCTGTC TCCAACCTCG	320
	GCTATGACCT GTACCGGGTG CGATCCAGCA TGAGCCCCAC	360
	GACCAACGTG CTCCTGTCTC CTCTCAGTGT GGCCACGGCC	400
25	CTCTCGGCCC TCTCGCTGGG AGCGGAGCAG CGAACAGAAT	440
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## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 418 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

## (ix) FEATURE:

- (A) NAME/KEY: CDS  
 (B) LOCATION: 117..1373  
 (D) OTHER INFORMATION: /note= "product =  
 "pigment epithelial-derived factor"  
 gene = "PEDF" codon\_start = 1"

## (ix) FEATURE:

- (A) NAME/KEY:  
 (B) LOCATION:  
 (D) OTHER INFORMATION: PEDF amino acid  
 sequence

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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25				30					35			
Gly	Ala	Leu	Val	Glu	Glu	Glu	Asp	Pro	Phe	Phe	Lys	
			40				45					
Val	Pro	Val	Asn	Lys	Leu	Ala	Ala	Ala	Val	Ser	Asn	
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85				90					95			
Glu	Gln	Arg	Thr	Glu	Ser	Ile	Ile	His	Arg	Ala	Leu	
			100					105				
Tyr	Tyr	Asp	Leu	Ile	Ser	Ser	Pro	Asp	Ile	His	Gly	
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Thr	Tyr	Lys	Glu	Leu	Leu	Asp	Thr	Val	Thr	Ala	Pro	
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Gln	Lys	Asn	Leu	Lys	Ser	Ala	Ser	Arg	Ile	Val	Phe	
		135				140						
Glu	Lys	Lys	Leu	Arg	Ile	Lys	Ser	Ser	Phe	Val	Ala	
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Pro	Leu	Glu	Lys	Ser	Tyr	Gly	Thr	Arg	Pro	Arg	Val	
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Leu	Thr	Gly	Asn	Pro	Arg	Leu	Asp	Leu	Gln	Glu	Ile	
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65

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Leu	Phe	Asp	Ser	Pro	Asp	Phe	Ser	Lys	Ile	Thr	Gly
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Lys	Pro	Ile	Lys	Leu	Thr	Gln	Val	Glu	His	Arg	Ala
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Gly	Phe	Glu	Trp	Asn	Glu	Asp	Gly	Ala	Gly	Thr	Thr
				365					370		
Pro	Ser	Pro	Gly	Leu	Gln	Pro	Ala	His	Leu	Thr	Phe
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Pro	Leu	Asp	Tyr	His	Leu	Asn	Gln	Pro	Phe	Ile	Phe
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Val	Leu	Arg	Asp	Thr	Asp	Thr	Gly	Ala	Leu	Leu	Phe
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## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 379 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (ix) FEATURE:
  - (A) NAME/KEY: Region
  - (B) LOCATION: 1..4

65/1

(D) OTHER INFORMATION: /note= "Met 1...Ile 4 is an N-terminal fusion to Asp 26...Pro 400 of SEQ ID NO:2; Met -18...Glu 25 of SEQ ID NO:2 is deleted"

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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			15					20				
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	25				30						35	
	Thr	Asn	Val	Leu	Leu	Ser	Pro	Leu	Ser	Val	Ala	Thr
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	Ala	Leu	Ser	Ala	Leu	Ser	Leu	Gly	Ala	Glu	Gln	Arg
	50						55					60
	Thr	Glu	Ser	Ile	Ile	His	Arg	Ala	Leu	Tyr	Tyr	Asp
				65						70		
10	Leu	Ile	Ser	Ser	Pro	Asp	Ile	His	Gly	Thr	Tyr	Lys
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	Glu	Leu	Leu	Asp	Thr	Val	Thr	Ala	Pro	Gln	Lys	Asn
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	Leu	Lys	Ser	Ala	Ser	Arg	Ile	Val	Phe	Glu	Lys	Lys
			100						105			
	Leu	Arg	Ile	Lys	Ser	Ser	Phe	Val	Ala	Pro	Leu	Glu
	110						115					120
15	Lys	Ser	Tyr	Gly	Thr	Arg	Pro	Arg	Val	Leu	Thr	Gly
				125						130		
	Asn	Pro	Arg	Leu	Asp	Leu	Gln	Glu	Ile	Asn	Asn	Trp
			135					140				
	Val	Gln	Ala	Gln	Met	Lys	Gly	Lys	Leu	Ala	Arg	Ser
	145					150					155	
	Thr	Lys	Gln	Ile	Pro	Asp	Glu	Ile	Ser	Ile	Leu	Leu
			160						165			
20	Leu	Gly	Val	Ala	His	Phe	Lys	Gly	Gln	Trp	Val	Thr
	170						175					180
	Lys	Phe	Asp	Ser	Arg	Lys	Thr	Ser	Leu	Glu	Asp	Phe
				185						190		
	Tyr	Leu	Asp	Glu	Glu	Arg	Thr	Val	Arg	Val	Pro	Met
			195				200					
25	Met	Ser	Asp	Pro	Lys	Ala	Val	Leu	Arg	Tyr	Gly	Leu
	205					210					215	
	Asp	Ser	Asp	Leu	Ser	Cys	Lys	Ile	Ala	Gln	Leu	Pro
			220						225			
	Leu	Thr	Gly	Ser	Met	Ser	Ile	Ile	Phe	Phe	Leu	Pro
	230						235					240
	Leu	Lys	Val	Thr	Gln	Asn	Leu	Thr	Leu	Ile	Glu	Glu
				245						250		
30	Ser	Leu	Thr	Ser	Glu	Phe	Ile	His	Asp	Ile	Asp	Arg
			255					260				
	Glu	Leu	Lys	Thr	Val	Gln	Ala	Val	Leu	Thr	Val	Pro
	265					270					275	
	Lys	Leu	Lys	Leu	Ser	Tyr	Glu	Gly	Glu	Val	Thr	Lys
			280						285			
	Ser	Leu	Gln	Glu	Met	Lys	Leu	Gln	Ser	Leu	Phe	Asp
	290					295						300
35	Ser	Pro	Asp	Phe	Ser	Lys	Ile	Thr	Gly	Lys	Pro	Ile
				305							310	

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Lys Leu Thr Gln Val Glu His Arg Ala Gly Phe Glu  
                   315                  320  
 Trp Asn Glu Asp Gly Ala Gly Thr Thr Pro Ser Pro  
 325                  330                  335  
 Gly Leu Gln Pro Ala His Leu Thr Phe Pro Leu Asp  
                   340                  345  
 5 Tyr His Leu Asn Gln Pro Phe Ile Phe Val Leu Arg  
       350                  355                  360  
 Asp Thr Asp Thr Gly Ala Leu Leu Phe Ile Gly Lys  
                   365                  370  
 Ile Leu Asp Pro Arg Gly Pro  
                   375

10 (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGYAAAYTTYT AYGAYCTSTA

20

20 (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

25 (ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTYTCYTCRT CSAGRTARAA

20

(2) INFORMATION FOR SEQ ID NO:6:

30

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

35

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(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Thr Ser Leu Glu Asp Phe Tyr Leu Asp Glu Glu Arg  
1 5 10  
Thr Val Arg Val Pro Met Met  
15

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Leu Tyr Tyr Asp Leu Ile Ser Ser Pro Asp Ile  
1 5 10  
His Gly Thr Tyr Lys Glu Leu Leu Asp Thr Val Thr  
15 20  
Ala Pro Gln Xaa Asn  
25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Met Asn Glu Leu Gly Pro Arg  
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4421 Base Pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Double  
(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Genomic DNA

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(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Human

(ix) FEATURE:  
(A) NAME/KEY: JT1  
(B) LOCATION:  
(C) IDENTIFICATION METHOD:  
(D) OTHER INFORMATION: 7.1 kb Bam HI  
fragment Derived from human placental  
genomic DNA; Also referred to as JT101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

10	GGATCCCTTG GTTGGGGTGT TGGGGAAGGC AGGGTTTTAA	40
	CGGAAATCTC TCTCCATCTC TACAGAGCTG CAATCCTTGT	80
	TTGATTACACC AGACTTTAGC AAGATCACAG GCAAACCCAT	120
	CAAGCTGACT CAGGTGGAAC ACCGGGCTGG CTTTGAGTGG	160
15	AACGAGGATG GGGCGGGAAC CACCCCAGC CCAGGGCTGC	200
	AGCCTGCCCA CCTCACCTTC CCGCTGGACT ATCACCTTAA	240
	CCAGCCTTTC ATCTTCGTAC TGAGGGACAC AGACACAGGG	280
	GCCCTTCTCT TCATTGGCAA GATTCTGGAC CCCAGGGGCC	320
20	CCTAATATCC CAGTTTAATA TTCCAATACC CTAGAAGAAA	360
	ACCCGAGGGA CAGCAGATTC CACAGGACAC GAAGGCTGCC	400
	CCTGTAAGGT TTCAATGCAT ACAATAAAAG AGCTTTATCC	440
	CTAACTTCTG TTA CTTCGTT CCTCCTCCTA TTTTGAGCTA	480
25	TGCGAAATAT CATATGAAGA GAAACAGCTC TTGAGGAATT	520
	TGGTGGTCCT CTACTTCTAG CCTGGTTTTTA TCTAAACACT	560
	GCAGGAAGTC ACCGTTTATA AGAACTCTTA GTTACCTGTG	600
30	TTGGATAAGG CACGGACAGC TTCTCTGCTC TGGGGGTATT	640
	TCTGTACTAG GATCAGTGAT CCTCCCGGGA GGCCATTTCC	680
	TGCCCCCATA ATCAGGGAAG CCTGCTCGTA AACAAACACAT	720
	GGACAGATAG GAGAGGCCAT TTGTAACCTA AGGAAACGGA	760

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	CCCGATACGT AAAGATTCTG AACATATTCT TTGTAAGGAG	800
	GTATGCCTAT TTTACAAAGT ACAGCCGGGT GTGGTGGCTC	840
	ATGGCTATAA TCCCAGCACT TTGGGAGGCC GAGGCGGGCG	880
5	GATCACCTGA GATCAGGAGT TTGAGACCAG CCTGACCAAC	920
	ACGGAGAAAC CCCGTCTGTA CTAAAAATAC AAAATTAGCA	960
	GGGTGTGGTG GTACATGCCT GTAATCCCAG CTACTGGGGA	1000
10	GGCTGAGGCA GGAGAATCAC TTGAACCCGG GAGGCGGAGG	1040
	TTGCAGTGAG CCGAGATCAC GCCATTGCAC TCCAATCTAG	1080
	GCAATAAGAG CAAAACTCCG TCTCAAACAA CAAAAACCA	1120
	AAGTATAACT GGGCTTTTTG AAGAACATGA AACATGCCCA	1160
15	GTGTCTGAAG TAGAATAACT ACCGAACTGT CCGTAGGACT	1200
	AAACTTTTTTC TTGAAAAAGC TCTACCAAAA AAAGTCACCG	1240
	GCCACTCCCT TGTCACAGTT ATTAGACAGG AGGAGAAATG	1280
	ATAATTCTAC TGCCCTTCAT TCTACAAATG TTTGAGTGCT	1320
20	AACTGTATTC CAGATTCTCA AAAAGCTATT GCCAGGTATC	1360
	TCTGGGGCTA CTGATTTCTT GATCATAATG CAATGGCAAC	1400
	CAACAGGCAC TTGGGCATGG TGAGGGTGGG CAAGCTTTCA	1440
	AAAGCAGCGT GGATCTGGCA TTCTTTTCCA CGAATGCACC	1480
25	TCAACTACTT GGCACCAGTG GTAACACAGC AACCAGGGTT	1520
	CCGACCTAGA GAATCCCGTA ACCTTCTGAC TGGAACGGGG	1560
	TCTGGGCTGT CGCTACACAT CCTGGTGGAA GGCAGCTATC	1600
	ATCCCTACCT TCTGCCTTCT GTCTCTTAAA TCTGAACCAC	1640
30	AAACAGCAAC GTCCATACCC TCAGCATTGT TAGAATCCCC	1680
	TGCAGCCTCC AGTTCTCATA CTGTCTGTAT TCTACTCGCC	1720
	AGTTTGGAGA GGTCTGGTGG AGAAAAGGAG TCTCTTTTCA	1760
35	GGCTTGACAA CAAATAGAAC TCAGGGCCGG GCGCGGTGGC	1800

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	TCACGCCTGT CATCCCAGCA CTGTGGGAGG CCGAAGCGGG	1840
	CGGATCACCT GAGGTCGGGA GCTCAAGACC AGCCTGGCCA	1880
	ACATGGAGAA ATCCCATCTT TACTAAAAAT ACAAATTAG	1920
5	CCGGGCGTAC TGGCGAATGC CTGTAATGCC AGCTTCTCGG	1960
	GAGGCTGAGG CAGGAGAATC GCTTGAACCT GGGAGGCAGA	2000
	GGTTGCGGTG AGCCAAGACT GTGCCACTGT ACTCCAGCCT	2040
10	TGGTGACAGA GGGAGACTCT GTCTTAAGAA AAAAAGAAAA	2080
	AAAAAAAAAA AGGGCCGGGC TCACGCCTGT AATCCCAGCA	2120
	CTTTGGGAGG CCAAATCACC TGAGGCCGGG AGTTTGATAC	2160
	CAACCTGACC AACATAGTGA AATCCCGTCT CTACTAAAAA	2200
15	TACAAAATTA GCCAGGCGTG GTGGCGGGCG CCTGTAATCC	2240
	CAGCTACTCG GGAGGCTGAA GCAGGAGAAT CACTTGAACC	2280
	CGGAAGGCGG AGGTTGCCGT AAGCCAAGAT CGCGCCATTG	2320
	CGCTCCAGCC TGGGCAACAA GAGTGAAACT CCATCTCAAA	2360
20	AACAAAACAA AACAAAACAA AACCAACAAC TCAGAAGGAG	2400
	GCATATGTGT TATAAAGTCT TTAATAACAAC TTTGATTTTA	2440
	TTAGTGTTTG GTTACTGACT CTGCCAAGAG TACAGAATGA	2480
	AGGGCAGAGA GTAAGGACTG GAAAACTGGC AGGAAACACA	2520
25	CTGACAGCCG TCATCCCTGG AGGAAACTGC TCAATAAAAC	2560
	GGCTCCATAT TTACTTCTCT GGTACAGTT CATACTCCAC	2600
	GATTTTAACA AAGGAGTCGA GGAAGCTAGA TACTGTAAGT	2640
	GGAACGGTGT GTCTCTGGAG GTAAGCAGGC TTGCTGATTT	2680
30	CTTGTTTTAT AATTCTTTTT TAATTACAAT GTAACACTA	2720
	AGAGCTTCAG TTCCCACTGG AGTGGTGCAC ACATCTCATT	2760
	ACTACTAAAA CCACAGGAAT GTTCCAGGGA AACAGACTAT	2800
35	CATCACTGAG CGAGGTGGAA TCCAGCCAAA ACCCCAGGCT	2840

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5	AACATCCAGA	TGCCTGCATA	TCAGCTAAAA	TCCTTTTAAA	2880
	GGACTTGGA	TCTCCAGATA	CTAGTTTAA	GTCTTTTCTG	2920
	GGAAGTGGGA	GTTTGTACTG	GAGGCCACTT	AACTATTTCA	2960
	AAAAATATTC	ACCAAAATAG	GTGTCTCTCT	GAAGTCAACG	3000
	GTTTGAGTCC	TCCTCAGCCC	TCATATCCTA	GGCTTCGGAC	3040
10	TGTTGGGAAA	GTCTTATCTT	CCTGACGAAA	GCTCAGCAGC	3080
	AACAGAACCT	GTTATTTTTT	TGTTGAGACA	GGGTCTTACT	3120
	CTGTCACCCA	GGCTGGAGTG	CAGTAGTGCG	ATCTTGGCTC	3160
	ACTGCAGCCT	CAGCCTACCA	GGCTCAGGTG	ACCCTATCTC	3200
	AGCTTCTCGA	GTAGGTGGGA	CTACAGGCAT	GTGCCACCAT	3240
15	GCTCGGTGAA	CTAAACAAAC	TTTTTTGTAG	TGATACGGTC	3280
	TCACTATATT	GCCCAGGCTG	GTTTTGAACT	CCTGGGCTCA	3320
	AGTGATCCTC	CCACCTCAGC	GTCTCAAAGT	ACTGGGATTA	3360
	CAGGTGTGAG	CCTCTACACT	GGGCCTGCAG	AACCTACACA	3400
	GAATCCGCAC	CTGGTCTGCA	GAACCCACAC	CCGACCCACA	3440
20	GAACCCACAC	CCGACCCACA	GAACCCACAT	CTGGCAGCAG	3480
	AACCTCTTAG	TATTTTTTTT	TTTTCTTTGA	GATGGAGTCT	3520
	GGCTCTGTCA	CCCAGGCTGG	AGTGCAGTGG	CGCGATCTCG	3560
	GCTCACTGCA	AGCTCTTCCT	CCCGGGTTCA	CCCCATTCTC	3600
	CTGCCTCAAC	CTCCCGAGTA	GCTGTGAATA	CAGGCGTCCG	3640
25	CCACCACGCC	CGACTAATTT	TTTTGTATTT	TTAGTAGAGA	3680
	CGGGGTTTCA	CCGTGTTAGC	CAGGATGGTC	TGGATCTCCT	3720
	GACCTCGTGA	TCTGCCTGCC	TCGGCCTCCC	AAAGTGCTGG	3760
	GATTACAGGC	TTGAGCCACC	GCACCCGGCC	TCTTATTTTT	3800
	TTTTTTGAGA	TGGAGTCTCA	CACTGTCACC	TGGGCTGGAG	3840
35	TGCAGTGGAG	CGATCTCGGC	TCACTGCAAC	CTCCGCCTCC	3880

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5 TGGGTTCAAG AGATTCTCCT GCCTCAGCCT CCCAAGTAGC 3920  
 TGGGATTACA GGTGCCCACC ACCACGCCTG GCTAGTTTTT 3960  
 TGTATTTTTTA GTAAAGATGG GGTTCACCA TGTGGCCAG 4000  
 5 GCTGGTCTTG AACTCCTGAC ATCAGGTGAT CCGCCCACCT 4040  
 TAGCCTCCCA AAGTGCTGGG ATTACAGGCG TGAGCCACCA 4080  
 TACCTGGCCA GCAAAACCTC TTAACTTGT GTTCCATGGG 4120  
 10 CTCCTTTTCT GTGGGTCAAA ATCCTCCTGG AACCTACAA 4160  
 TGCAGGCCCT ACAGGGGTGG GTGGTAAGTC CAACAAACAG 4200  
 GATTCATCT TCTGGAGCTC CTGGATTTC TCGTCCCATG 4240  
 GGCCACAGTG CAGCGACAGA ACCTCCTCAG CTTTCTGTAT 4280  
 15 TGTGCTCAGG GCTTCGGGTA CTGCAAACCT GAGCCAAGGG 4320  
 AGGTAAGAGG AGTTAGTTCA CTGATTCGTG AGGCAAATGT 4360  
 TAATTGAGGG CCTACTCACA CACCGTGAAG AATGTAAGAT 4400  
 CATTTCTGTC ATCAAGGATC C 4421

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## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 7210 Base Pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Double  
 25 (D) TOPOLOGY: Unknown  
  
 (ii) MOLECULE TYPE: Genomic DNA  
  
 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Human  
  
 (vii) IMMEDIATE SOURCE:  
 30 (A) LIBRARY:  $\lambda$ DASH II  
  
 (ix) FEATURE:  
 (A) NAME/KEY: JT6A  
 (B) LOCATION:  
 (C) IDENTIFICATION METHOD:  
 35 (D) OTHER INFORMATION: 7.0 kb Not 1-Not  
 fragment; Derived from human placental  
 genomic DNA; also referred to as JT106

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

	GATCTAGAGC GGCCGCAGGG TGGACTGTGC TGAGGAACCC	40
	TGGGCCCAGC AGGGGTGGCA GCCCGCGCAG TGCCACGTTT	80
5	GGCCTCTGGC CGCTCGCCAG GCATCCTCCA CCCCCTGGTC	120
	CCCTCTGACC TCGCCAGCCC TCCCCCGGGA CACCTCCACG	160
	CCAGCCTGGC TCTGCTCCTG GCTTCTTCTT CTCTCTATGC	200
10	CTCAGGCAGC CGGCAACAGG GCGGCTCAGA ACAGCGCCAG	240
	CCTCCTGGTT TGGGAGAAGA ACTGGCAATT AGGGAGTTTG	280
	TGGAGCTTCT AATTACACAC CAGCCCCTCT GCCAGGAGCT	320
	GGTGCCCGCC AGCCGGGGGC AGGCTGCCGG GAGTACCCAG	360
15	CTCCAGCTGG AGACAGTCAG TGCCTGAGGA TTTGGGGGAA	400
	GCAGGTGGGG AAACCTTGGC ACAGGGCTGA CACCTTCCTC	440
	TGTGCCAGAG CCCAGGAGCT GGGGCAGCGT GGGTGACCAT	480
	GTGGGTGGGC ACGCTTCCCT GCTGGGGGTG CAGGGGGTCC	520
20	ACGTGGCAGC GGCCACCTGG AGCCCTAATG TGCAGCGGTT	560
	AAGAGCAAGC CCCTGGAAGT CAGAGAGGCC TGGCATGGAG	600
	TCTTGCTTCT TGCAAACGAG CCGTGTGGAG AGAGAGATAG	640
	TAAATCAACA AAGGGAAATA CATGGTCTGT CCGAGGATGA	680
25	GCTGCCGGAG AGCAATGGTG AAAGTGAAGT GGGGGAGGGG	720
	GCGGGGCTGG GAGGAAAAGC CTTGTGAGAA GGTGACACGA	760
	GAGCACGGCC TTGAAGGGGA AGAAGGAGGG CACTATGGAG	800
	GTCCCGGCGA AGCGTGGCCT GGCCGAGGAA CGGCATGTGC	840
30	AGAGGTCCTG CCGAGGAGCT CAAGACAAGT AGGGGACGGT	880
	GGGGCTGGAG TGGAGAGAGT GAGTGGGAGG AGGAGTAGGA	920
	GTCAGAGAGG AGCTCAGGAC AGATCCTTTA GGCTCTAGGG	960
35	ACACGATAAA CACAGTGTTT TTTGTCTTGT CAAGTGTGTC	1000

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5	CTTTTTTATTT TTTTGAAAGA GTCTCGCTCT GTAGCCCAGG	1040
	CTGGAGTGCA GCGGTGCGAC CTCGGCTCAC TGCAACCTCT	1080
	GCCTCCCGGG TCCAAGCAAT TCTCCTGCCT CAGCCTCCCG	1120
	AGTAGCTGGG ATTACAGGCA CCCGCCACCA CGCACTGCTA	1160
	ATTTTTTGAT TTTAGTAGAG ACCGGGTTTT GCCATGTTGG	1200
10	TCAGGCTGGT CTCGAACTCC TGACCTCAGG TGATCCGCCC	1240
	GCCTCGGCCT CCCAGAGTGG TGTGAGCCAC TATGCCCTGC	1280
	AGCACTTGTC AAGTCTTTCT CAGCGTTCCC CTCCTCTCCA	1320
	CTGCAGCTCC CAGTGCCCCA GTCTGGGCCT CGTCTTCACT	1360
	TCCTGGGATC CCTGACATTG CCTGCTAGGC TCTCCCTGTC	1400
15	TCTGGTCTGG CTGCCTTCAC TGTAACCTCC ACCCAGCAGG	1440
	TACCTCTTCA GCACCTCCCA TGAACCCAGC AGAATACCAA	1480
	GCCCTGGGGA TGCAGCAACG AACAGGTAGA CGCTGCACTC	1520
	CAGCCTGGGC GACAGAGCAA GACTCCGCCT GAAGAAAAAA	1560
	AAAAGGACCA GGCCGGGCGC GGTGGCTCAC GCCTGTAATC	1600
20	CCAGCACTTT GGGAGGCCGA GGTGGGTGGA TCATGAGGTC	1640
	AGGAGTTCAA GACCAGCCTG GCCAAAATGG TGAAACCCCG	1680
	TCTCTACTGA AAAATACAAA AATTAGCTGG GTGCAGTGGC	1720
	GGGCGCCTGT AGTCTCAGCT ACTCAGGAGG CTGAGGCAGG	1760
	ATAATTGCTT GACCCCAGGA GGCAGAGGTT GCAGTGAACC	1800
25	GAGATCACGC CACTGCACTC CAGCCTGGGC GACAGAGCAA	1840
	GACTCTGCCT CAAAAAAAAG AATAAAAATA AAAAAAAGGA	1880
	CCAGATACAG AAAACAGAAG GAGACGTACT ATGAAGGAAA	1920
	TTGGAGAGCT TTTGGGATAC TGAGTAACTC AGGGTGGCCT	1960
	TTCCCAGGGG ACATTTAGCT GAGAGATAGA CGGTATGAAG	2000
35	ACCTGACCGT TCAGAAACAG GGGAAGAGGC AGCAGCCCGG	2040

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5	GCAAAGGCCT TTGGGGCAGG AAAGGGCTTG GATCACTGGA	2080
	GAAGCAGAAA GATGGCCAGT GTGACCAGAG TGTGACAAAG	2120
	TCAGAGAAAA CCAGGAAGAT GGAGCTGGAG ACACAGGCGG	2160
	GGCCAGATCA CGAGGGTCCT CGCAGACCAG AGCAAGGGTT	2200
	TGGATTTTAT TCCAAGTATG AAGGGAAGCT GCTGAAGTGT	2240
10	GTTTTCTTTT ACAATTTGTA GTTGAAATAT AATATGCAAA	2280
	GTACACAAGT CTTAACTATA TGTAAGCTTA ATGAATGTTT	2320
	CCATGAACCA AATACCGCTG TGCAACCATC ACCAGCTCAA	2360
	GAGACGAACC CTTCTCCCTC CTCCTGACTG CCAGTAACAT	2400
	AGTGGTTCAG CTCAAGAAAC AGAACTCTTC TGACTTCCCC	2440
15	TAACATAGCG GGTTTTCTTT TTTGTTTTGT TTTTGTGTGT	2480
	TTTTTAAGAG ACAATGTCTT TATTATTTTT ATTTTTTTTT	2520
	ATTTTTGAGA CGGAGTCTTG CTGTGCCCCA GGCTGGAGTG	2560
	CAGTGGTGCG ATCTCGGCTC ACTGCAGGCT CTGCCCCCGG	2600
	GGGTTCATGC CATTCTCCTG CCTCAGCCTC CCTAGCAGCT	2640
20	GGGACTACAG GTGCCCCCA CCTCGCCCGG CTATTTTTTT	2680
	GTATTTTTAG TGGAGACGGG GTTTCACCGT GTTAGCCAGG	2720
	ATGGTCTCGA TCTCCTGACC TCGTGATCCG CCCACCTCGG	2760
	CCTCCCAAAG TGCTGGGATT ACAGGCATGA GCCACGCGC	2800
	CCAGCCAAGA GACACGGTCT TGCTCTGTCTG CCCAGGCTGG	2840
25	ATGGAGTGCC GTGGTGCGAT CACAGCTCGC GGCAGCCTTG	2880
	ACATCCTGGG CTCAAGCAAC CTTCTGCCT TGGCCTCCCA	2920
	AATGTTGGGA TTATAGGCAT GAGCCACTGT GCTTGGCATC	2960
	TATTCATCTT TAATGTCAAG CAGGCAATTG AATATTTGAT	3000
	CAGGGATAGA ATTGTCTATT TGGGGGTATG CAGATGTGCT	3040
35	TCATGTCATG GAACTGGGCC GGGCGCGGTG GCTCATGCCT	3080

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5	ATAATCCCAG	CACTTTGGGA	GGCCGAGGCA	GGCGGATCAT	3120
	AAGGTCAGGA	GATCGAGACC	ATCCGGGCCA	ACACGGTGAA	3160
	ACCCCGTCTC	TACTAAAAAT	ACAAAAATTA	GGCAGGTGTG	3200
	GTGGTGCGTG	CCTGTAGTCC	CAGCTACTCA	GGGAGGCTGA	3240
	GACAGGAGAA	TTGATTGAAC	CTGGGAGGCA	GAGGTTGTAG	3280
10	TGAGCCAAGA	TCGCGCCACT	GCACTCCAGC	CTGGGCGACA	3320
	TGAGCGAGAC	TCCGTCTCAA	AAATAAACAA	AAAAAAGTCA	3360
	TGGAATTGAT	GGAAATTGCC	TAAGGGGAGA	TGTAGAAGAA	3400
	AAGGGGTCTC	AGGATCAAGC	CAGCAGAGAA	GGCAGAAAAG	3440
	GTAAGGTGTG	TGAGGTGGCA	GAAAAAGGGA	AGAGTGTGGA	3480
15	CAGTGAGGGT	TTCAAGGAGG	AGGAACTGTC	TACTGCCTCC	3520
	TGCCAAGGAC	GGAGGTGTCC	ACTGCCAGTT	GACATAAGGT	3560
	CACCCATGAA	CTTGGTGACA	GGAATTTTCAG	TGGAGAAGTG	3600
	GCCACAGACA	CAAGTCTAGA	ATTGAAATGG	GAGCCGAGGC	3640
	AGCGTAGACA	AAAGAGGAAA	CTGCTCCTTC	CAGAGCGGCT	3680
20	CTGAGCGAGC	ACCGAGAAAT	GGGCAGTGGC	TTTAGGGGAT	3720
	GTAGCGTCAA	GGAAGTGTCT	TTTAAAGAAG	TCGGGGGCCG	3760
	GGCACGGTGG	CTCACGCCTG	TAGTCCCAGC	ACTTTGGGAG	3800
	GCCGAGGCAG	GCAGATCACT	TGAGGTCAGG	AGTTCGAGAC	3840
	CAGCCTGGCT	AACACGATGA	AACCCCGTCT	CTACTAAAAA	3880
25	TACAAAAAAT	TAGCTGGGCA	CGGTGGCTCG	TGCCTGTAAT	3920
	CCCAGCACTT	TGGGAGGCAG	AGGTGGGCAG	ATCACTTGAG	3960
	GTCAGGAGTT	TGAGACCAGC	CTAGCCAACA	TGGTGAAACC	4000
	CCATCTCTAC	TAAAACTACA	AAAATTAGCC	GGGAGTGGTG	4040
	GCACGTGCCT	GTAATCCCAG	CCAGTCAGGA	GGCTGAGGCA	4080
35	GGAGAATCAC	TGGAATCCTG	GAGGTGGAGG	TGGCAGTGAG	4120



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	CCGAGATGGT ACCTCTGTAC TCCAGCCTGG GGGACAGAGT	4160
	GAGACTCCGT CTCAAAAAAA AAAGAAGGTG GGGAAGGATC	4200
	TTTGAGGGCC GGACACGCTG ACCCTGCAGG AGAGGACACA	4240
5	TTCTTCTAAC AGGGGTCGGA CAAAAGAGAA CTCTTCTGTA	4280
	TAATTTATGA TTTTAAGATT TTTATTTATT ATTATTTTTT	4320
	ATAGAGGCAA GCATTTTTTCA CCACGTCACC CAGGCTGGTC	4360
10	TCCAACCTCT GGGCTCAAGT GTGCTGGGAT TATAGCCATG	4400
	AGTCACCACA CCTGGCCCAG AAACCTTTACT AAGGACTTAT	4440
	TTAAATGATT TGCTTATTTG TGAATAGGTA TTTTGTTTAC	4480
	GTGGTTCACA ACTCAAAAGC AACAAAAAGC ACCCAGTGAA	4520
15	AAGCCTTCCT CTCATTCTGA TTTCCAGTCA CTGGATTCTA	4560
	CTCTTGGGAT GCAGTGTTTT TCATCTCTTT TTTGTATCCT	4600
	TTTGGAATA GTATTCTGCT TTAAAAAGCA AATACAGGCC	4640
	AGGTATGGTG GCTCACTCCT GTAATCCCAG CACTTTGGGA	4680
20	GCCGAGGCAG GTGATCACCT AAGGTCAGGA GTTCAAGACC	4720
	AGCCTGGCCA ATATGGTGAA ACCCTGTCTG TACCAAACA	4760
	CAAAAACAAA AACAAAAACA AAAATTAGCC GGGCGTGGTG	4800
	GCGTGCTCCT GTAATCCCAG CTAATCAGGA GGCTGAGGCA	4840
25	GGAGAATCGC TTGAACCTGG GAGGCAGAGG TTGCAGTGAG	4880
	CCGAGATTGT GCCACTGTAC TCCAGCCTGG GCCACAGAGC	4920
	AAGGTTCCAT CTCAAACAAA ACAAACAAA ACAACAAAA	4960
	AAACAAAACA AAAGCTAATA CAAACACATA TACAATAGAC	5000
30	AAAACGTGTA ATATTTTATT ATTTTATTTT TTTTTAGTAG	5040
	AGACAGGGTT TCACCATGTT GGCCAGGATG GTCTCAAAC	5080
	CCTGACCTCA GGTGATCCAC CCACCTCAGC CTCCCGATAG	5120
35	TTAGGATTAC AGGCATGAGC CACCACACCC GGCCTAAAT	5160

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5	TGTAAACGTT TTAGAAGAAA GTATAGATGA ATCCCTTCGT	5200
	GATCTCGGGG AAGAAGAGAT TTTTAAAAA AGATACCAA	5240
	AGAAGCACAA ATTATAAAG AAAAGATTGA AAATGTTGGT	5280
	GTTAAAATTA AAAACTTGTT TTAACAAG CTTGTGTAAC	5320
	CCATGACCCA CAGGCTGCAT GTGGCCAGAA AAAGCTTTGA	5360
10	CTGCAGCCCA ACACAAATTC GTAAACTTTC CTAAAACATT	5400
	ATGAGATTTT TTTTGAGATT TTGTTTGTGTT TTGTTTTTTG	5440
	TTTTTTTAGC TCATTCGGTA TCATTAATGT TAGCATATTT	5480
	TACGTGGGGC CCAAGACAAT TCTTCTTCCA ATGTGTCTCA	5520
	GGGGAGCCAA AAGATTGGAC ACCCTGCCA TAAACATGAA	5560
15	AAGACAATGG CCGGGCACGG TGGCTCACGC CTGTAATCCC	5600
	AGCACTTTGG GAGGCTGAGG GGGGCGGGAT CACCTGAGGT	5640
	CAGGAGTTTG AGACAAGCGT GACCAATGTG GTGAAACCCT	5680
	GTCTCTACTA AAAATACAAA AATTAGCCGG GCATGCTCGT	5720
	GCACACCTAT AGTCCCAACT ACTCAGCAGG GTGAGGCAGG	5760
20	AGAACCTCTT GAACCCGGGA AGCGGAGGTT GCAGTGAGCC	5800
	GACATTGCAC CCCTGCACTC CAGCCTGGGT GACAGAGTGA	5840
	GTCTCCACTG GAAAAAAAAA AAAAGAACA GTGTGATACA	5880
	TTGACCTAAG GTTTAAGAAC ATGCAAACTG ATACTATATA	5920
	TCACTTAGGG ACAAAAACCTT ACATGGTAAA AGTAAAAAGA	5960
25	AATGTACGAA AATAATAAAA ATCAAATTCA AGATGGTGGT	6000
	TATGGTGACG GGAAAGAACT GAGGCGGAAA TATAAGGTTG	6040
	TCACTATATT GAGAAATTTT TCTATCTTTT TTTCTTTTTT	6080
	CTTTTTTTTGA GACGGGGTCT CGCTCTGTCTG CCCAGGATGG	6120
	AGTGCAGTGG TGTGATCTCA GCTCACTGCA ACCTCCGCCT	6160
35	CCCAGGTTTA AGTGATTCTC CTGCCTCAGA CTCCAAGTA	6200

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5	GCTGGGACTA CAGGTGCGCG CCAACACACC TGGGTAATTT	6240
	TGTTTGTATT TTTAGTAGAG ATGGGGTTTC ACCGTGTTGA	6280
	CTAGGCTGGT CTCGAACTCC TGACCTCAGG TGATCCCCCG	6320
	GCCTCGGTCT CCCAAAGTGC TGGGATAACA AGCGTGAGCC	6360
	ACTGCGCCCA GCTTTGTTTG CATTTTtagg TGAGATGGGG	6400
10	TTTCACCACG TTGGCCAGGC TGGTCTTGAA CTCCTGACCT	6440
	CAGGTGATGC ACCTGCCTCA GTCTCCCAA GTGCTGGATT	6480
	ACAGGCGTTA GCCCCTGCGC CCGGCCCTG AAGGAAAATC	6520
	TAAAGGAAGA GGAAGGTGTG CAAATGTGTG CGCCTTAGGC	6560
	GTAATGGATG GTGGTGCAGC AGTGGGTAA AGTTAACACG	6600
15	AGACAGTGAT GCAATCACAG AATCCAAATT GAGTGCAGGT	6640
	CGCTTTAAGA AAGGAGTAGC TGTAATCTGA AGCCTGCTGG	6680
	ACGCTGGATT AGAAGGCAGC AAAAAAGCT CTGTGCTGGC	6720
	TGGAGCCCCC TCAGTGTGCA GGCTTAGAGG GACTAGGCTG	6760
	GGTGTGGAGC TGCAGCGTAT CCACAGGTAA AGCAGCTCCC	6800
20	CTGGCTGCTC TGATGCCAGG GACGGCGGGA GAGGCTCCCC	6840
	TGGGCTGGGG GGACAGGGGA GAGGCAGGGG CACTCCAGGG	6880
	AGCAGAAAAG AGGGGTGCAA GGGAGAGGAA ATGCGGAGAC	6920
	AGCAGCCCCCT GCAATTTGGG CAAAAGGGTG AGTGGATGAG	6960
	AGAGGGCAGA GGGAGCTGGG GGGACAAGGC CGAAGGCCAG	7000
25	GACCCAGTGA TCCCCAAATC CCACTGCACC GACGGAAGAG	7040
	GCTGGAAAGG CTTTtGAATG AAGTGAGTGG GAAACAGCGG	7080
	AGGGGCGGTC ATGGGGAGGA AAGGGGAGCT AAGCTGCTGG	7120
	GTCGGGTCTG AGCAGCACCC CAAGACTGGA GCCCGAGGCA	7160
	AGGAGGCTCA CGGGAGCTGC TTCCACCAAG GGCAGTCAGG	7200
35	AAGGCGGCCG	7210

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## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1988 Base Pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Double  
 (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Genomic DNA

## (vi) ORIGINAL SOURCE:

- 10 (A) ORGANISM: Human

## (ix) FEATURE:

- (A) NAME/KEY: JT8A  
 (B) LOCATION:  
 (C) IDENTIFICATION METHOD:  
 (D) OTHER INFORMATION: 2 kb PCR product using  
 15 primers, SEQ ID: 13 and 14; Also referred  
 to as JT108

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

	ACAAGCTGGC AGCGGCTGTC TCCAACCTCG GCTATGACCT	40
	GTACCGGGTG CGATCCAGCA NGAGCCCCAC GACCAACGTG	80
20	CTCCTGTCTC CTCTCAGTGT GGCCACGGCC CTCTCGGCCC	120
	TCTCGCTGGG TGAGTGCTCA GATGCAGGAA GCCCCAGGCA	160
	GACCTGGAGA GGCCCCCTGT GGCCTCTGCG TAAACGTGGC	200
	TGAGTTTATT GACATTTTCAG TTCAGCGAGG GGTGAAGTAG	240
25	CACCAGGGGC CTGGCCTGGG GGTCCCAGCT GTGTAAGCAG	280
	GAGCTCAGGG GCTGCACACA CACGATTCCC CAGCTCCCCG	320
	AAAGGGGCTG GGCACCACTG ACATGGCGCT TGGCCTCAGG	360
	GTTTCGCTTAT TGACACAGTG ACTTCAAGGC ACATTCTTGC	400
30	ATTCCTTAAC CAAGCTGGTG CTAGCCTAGG TTCCTGGGAT	440
	GTAAGTGCAA ACAAGCAGGT GTGGGCTTGC CCTCACCGAG	480
	GACACAGCTG GGTTTCACAGG GGAACATAA CCAGCTCACT	520
35	ACAGAATAGT CTTTTTTTTT TTTTTTTTTN NNCTTTCTGA	560

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	GACGGAGTCT	CGCTTTGTCN	CCAAGGCTGG	AGTGCAGTGG	600
	TGTGATCTCA	GCTCACTGCA	ACCTCTGCCT	CCCTGGTTCA	640
	AGGAATTCTC	CTGCCTCAGC	CTCCAGAGTA	GCTGGGATTA	680
5	CAGGCACCTG	CCATCATGCC	CAGCTAATTT	TTGTATTTTT	720
	AGTAGAGACG	GGGTTTCACC	ATGTTGCCTA	GGCTGGTCTC	760
	AAACTCCCGG	GCTCAAGCGA	TCCACCCGCC	TTGGCCTCCC	800
10	AAAGTGCTGG	GATTACAGGC	GTGAGCCACC	GCGCCTGGCC	840
	AGAATAATCT	TAAGGGCTAT	GATGGGAGAA	GTACAGGGAC	880
	TGGTACCTCT	CACTCCCTCA	CTCCCACCTT	CCAGGCCTGA	920
	TGCCTTTAAC	CTACTTCAGG	AAAATCTCTA	AGGATGAANA	960
15	TTCCTTGGCC	ACCTAGATTG	TCTTGAAGAT	CAGCCTACTT	1000
	GGGCTCTCAG	CAGACAAAAA	AGATGAGTAT	AGTGTCTGTG	1040
	TTCTGGGAGG	GGGCTTGATT	TGGGGCCCTG	GTGTGCAGTT	1080
	ATCAACGTCC	ACATCCTTGT	CTCTGGCAGG	AGCGGAGCAG	1120
20	CGAACAGAAT	CCATCATTCA	CCGGGCTCTC	TACTATGACT	1160
	TGATCAGCAG	CCCAGACATC	CATGGTACCT	ATAAGGAGCT	1200
	CCTTGACACG	GTCACTGCCC	CCCAGAAGAA	CCTCAAGAGT	1240
	GCCTCCCGGA	TCGTCTTTGA	GAAGAGTGAG	TCGCCTTTGC	1280
25	AGCCCAAGTT	GCCTGAGGCA	TGNGGGNTCC	ATGCTGCAGG	1320
	CTGGGGGGGT	CTTTTTTTTT	TTTTTNNNNA	GACGGAGTCT	1360
	CGCTCTGTTG	CCCAGGCTGG	AGTGCAGTGG	CGNGATCTCG	1400
	GCTCACTGCA	ACCTCCACCT	CCCGGGTTCA	CACCATCCTC	1440
30	CTGCCTCAGC	CTCCCGAGTA	GCTGGGACTG	CAGGNGCCCA	1480
	GCTAATCTTT	NTTGTATTTT	TAGCAGAGAC	GGGGTTTCAC	1520
	CGTGTTTGCC	AGGATAGTCT	CGATCTCCTG	ACCTGGTGTT	1560
35	CTGCCCCCCT	CGACCTCCCA	AAGTGCTGGG	ATTACAGGTG	1600

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TGAGCCACCG CGCTCGGCCC GTTCTAAAC AATAGATCAT 1640  
 GTGTGCCCAG GCCTGGCCTG GCACTGGTGT GGAGGAAGGG 1680  
 5 CCCGTGAGCC CAAAGAGGCT CAGAAAGAGG AAGTGGGCTG 1720  
 CAGGAGACGG TGGGAGGGGC NGGGAGGGCA GTGGCGCGAT 1760  
 GTGGGGAAAT CTGCTGCCCC CCTGGCCAGT GCCTGGGGAT 1800  
 GCCAGCAGAA GTCCTGGCAA GTCACAGGAA GATGCTGGCT 1840  
 10 GGGAAGTCAG GGCCTGCTGA GCGCTAAACC AGAACCCGAG 1880  
 CCTGGCAGGC TCTCAAAGAC GGGATGCTTG TCGTNGAGTC 1920  
 TCATANGCTA ACCTCTGCTC CGCCTCTTCT CAGAGCTGCG 1960  
 CATAAAATCC AGCTTTGTGG CACCTCTG 1988

15

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3267 Base Pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Double  
 20 (D) TOPOLOGY: Unknown  
  
 (ii) MOLECULE TYPE: Genomic DNA  
  
 (ix) FEATURE:  
 (A) NAME/KEY: JT109  
 (B) LOCATION:  
 (C) IDENTIFICATION METHOD:  
 25 (D) OTHER INFORMATION: 3.3 kb PCR product  
 using primers, SEQ ID No: 15 and 16

25

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GATTCCAGCT TTGTGGCACC TCTGGAAAAG TCATATGGGA 40  
 30 CCAGGCCCAG AGTCCTGACG GGCAACCCTC GCTTGGACCT 80  
 GCAAGAGATC AACAACTGGG TGCAGGCGCA GATGAAAGGG 120  
 AAGCTCGCCA GGTCCACAAA GGAAATTCCC GATGAGATCA 160  
 GCATTCTCCT TCTCGGTGTG GCGCACTTCA AGGGTGAGCG 200  
 35 CGTCTCCAAT TCTTTTTTCAT TTATTTTACT GTATTTTAAAC 240

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	TAATTAATTA ATTCGATGGA GTCTTACTCT GTAGCCCTAA	280
	CTGGAGTGCA GTGGTGCGAT CTCAGCTCAA TGCAACCTCC	320
5	GCCTCCCAGG TTCAAGCAAT TCTTGTGCCT CAGCCTCCCG	360
	AGTAGCTGGG ATTACAGGGA TGTACCACCA CTCCCGGCTA	400
	ATTTTTTTGTA TTTAATAGAC ATGGGGTTTC ACCATGTTGG	440
	CCAGGCTGGT CTCGAACTCC TGAGCTCAGG TGGTCTGCCC	480
10	GCCTCAGCCT CCCAAAGTGC TAGGATTACA AGCTTGAGCC	520
	ACCACGCCCA GCCCTTTTTA TTTTAAATT AAGAGACAAG	560
	GTGTTGCCAT GATGCCCAGG CTGGTCTCGA ACTCCTGGGC	600
	TCAAGTAATC CTCCCACCTT GGCCTCCCAA AGTGCTGGGA	640
15	TTACAGGCAT GAGCCACCGC GCCCGGCCCT TTTACATTTA	680
	TTTATTTATT TTTTGAGACA GAGTCTTGCT CTGTCACCCA	720
	GGCTGGAGTG CAGTGGCGCG ATCTCGGCTC ACTGCAAGCT	760
	CTGCCTTCCA GGTTACACACC ATTCTCCTGC CTCGACCTCC	800
20	CGAGTAGCTG GGACTIONAGG CGCCCGCCAC TGCGCCCTAC	840
	TAATTTTTTG TATTTTTAGT AGAGACGGGG TTTCACCGTG	880
	GTCTCGATCT CCTGACCTCG TGATCCACCC GCCTCAGCCT	920
	CCCAAAGTGC TGGGATTACA GGCCTGAGCC ACTGCGCCCG	960
25	GCCCTTTTAC ATTTATTTTT AAATTAAGAG ACAGGGTGTC	1000
	ACTATGATGC CGAGGCTGGT CTCGAACTCC TGAGCTGAAG	1040
	TGATCCTCCC ACCTCGGCCT CCCAAAATGC TGGGATTACC	1080
30	ATGTCCAACCT TTCCACTTCT TGTGTGACCA AGGATGGATG	1120
	GCAGACATCA GAAGGGGCTT GGAAAGGGAG GTGTCAAAGA	1160
	CCTTGCCCAG CATGGAGTCT GGGTCACAGC TGGGGGAGGA	1200
	TCTGGGAACT GTGCTTGCTT GAAGCTTACC TGCTTGTCAT	1240
35	CAAATCCAAG GCAAGGCGTG AATGTCTATA GAGTGAGAGA	1280

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	CTTGTGGAGA CAGAAGAGCA GAGAGGGAGG AAGAATGAAC	1320
	CTGGGTCTGT TTGGGGCTTT CCCAGCTTTT GAGTCAGACA	1360
5	AGATTTATTT ATTTATTTAA GATGGAGTCT CATTCTGTTG	1400
	CCCAGGCTGG AGTGCAGTGG TGCCATCTTG GCTCACTACA	1440
	GCCTCCCCAC CTCCCAGGTT CAAGTGCTTC TCCTGCCTCA	1480
	GCCTCCCGAG TAGTTGGGAT TACAGGCGCC CGCCACCACA	1520
10	CCCAGCTAAT TTTTGTATTT TCAGTAGAGA TGGGGTTTCG	1560
	CCATGCTGGC CAGGCTGTTC TCGAAAACTC CTGACCTCAG	1600
	ATGATCCACC CGCCTCGGCC TCCCACAGTG CTGGGATTAC	1640
	AGGCGTGAGC CACTGCGCTG GCCAAATCAG ACAAGGTTTA	1680
15	AATCCCAGCT CTGCCTGTAC TAGCTGAGGA ACTCTGCACA	1720
	CATTTCATAA CCTTTCTGGG CCTACGTTCT CACCTTTAAC	1760
	GTGAGGATAA TATATCTACT TCATAGACAC CTTTTTATGT	1800
	TGTCTCCAAG TTTTCTAACA GCTCTAGTTC TGTACCCAAG	1840
20	ACATGGCAGG TGGCCAACGA CATCCTTCTA GGCTGTGGTG	1880
	ATGTGTTTGG AGCTTGTTCC ACGGGTCTTG TGTGGGGCCA	1920
	GCCCTGTTCA GATAAGGCCT TGTGGGGTGG CCTGGGGTAG	1960
	GGGGAGGGGT TGGGCAAACT CTCCCTTAAA ACGCTTTGTA	2000
25	ACCATCTGAG GCACCAGCAA GAGCGGCCCC CGAGCCTGGA	2040
	CAAAATCCAA ACGGCTTCCT ACTTCAAGCA CTGATGTCTA	2080
	GTGAGTGAAG GAACAGCTCT GGGTCCAGGA TATTATAGGT	2120
30	CACATTAAAC TAAAGGGGCT TGGCCATCAG CTGGCTTCCA	2160
	GAGCGTCAGC CAGTTACTTC ACCTCTTTGG CTTTGGCCTG	2200
	TTTTCAGCTA CAAGAGGACT TAATCCAGAG GACCTCAGAG	2240
	GTCCTTCCCA GCTCAGACCT TCTTTGACTG TCTCCCAGAG	2280
35	AACTGCTGT AGGAGTGCAC ACCAGTTTAC TTTTCTTTCT	2320



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5 TTTGTTTTTG AGATGGAGTT TCGCTCTTTT TGCCTAGGCT 2360  
 GGAGTGCTGT GGTGTGATCT CAGCTCACTG CAACCTCTGG 2400  
 CTCCCAGGTT CAAGTGATTC TCCTGTCTCT GCCTCCCGAG 2440  
 TAGCTGGGAT TACAGACACC CACCACTGCA CCCGGCTAGT 2480  
 TTTTGTATTT TCAGTAGAGA TGGGGTTTCG CCATGCTGGC 2520  
 CAGGCTGTTC TCGAAAACCTC CTGACCTCAG ATGATCCATC 2560  
 10 CGCCTTGGCC TCCCAAAGTG CTGAGATTAC AGATGTGAGG 2600  
 CACCACACCC GGCCATTTTT GTATTTTTAG TAGAGACGGG 2640  
 GTTTTGCCAT GTTGGCCACG CTGGTCTCAA ACTCCTGACC 2680  
 TCAAGTGATC TGCCACCTT GGCCTCCTGA AGGGCTGGGA 2720  
 15 CTACAGGCGT GAGTCACCGT GCCCGGCCAT TTTTGTATTT 2760  
 TTAGGACAGC GTTTTTTCAT GTTGGCCAGG CTGGTCTCAA 2800  
 ACTCCTGACC TCAAGTGATC CACCCACCCC GGCCTCCCAA 2840  
 TATGCTGGGA TTCCAGGTGT GAGTTACCAT GCCCGGCTAC 2880  
 20 CACTTTACTT TTCCTGCAGG CTATCACAGA ACGTGTACAA 2920  
 TCTAGACTCT AATCAACCAA ATCAACGTCT TGCCATCGGA 2960  
 GTTTGCTGGT GAAGGGCACT TGGGGTCCTG GAAATAACTG 3000  
 TAGGCTCCAA GCCACACACA CTGAGATAGG CCTATTCCCT 3040  
 25 GAGGCCTCAG AGCCCCTGAC AGCTAAGCTC CCTTGAGTCG 3080  
 GGCAATTTTC AACAACTGTC TCTGGGGACA CAGCATGGCG 3120  
 CCACTGTCTT TCTGGTCTCC TGGGGCTCAG ACTATGTCAT 3160  
 30 ACACTTCTTT CCAGGGCAGT GGGTAACAAA GTTTGACTCC 3200  
 AGAAAGACTT CCCTCGAGGA TTTCTACTTG GATGAAGAGA 3240  
 GGACCGTGAG GGTCCCCATG ATGAATC 3267

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## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Unkown
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Oligonucleotide

## (ix) FEATURE:

- (A) NAME/KEY: 603
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: primer in a polymerase chain reaction

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACAAGCTGGC AGCGGCTGTC

20

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Unkown
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Oligonucleotides

## (ix) FEATURE:

- (A) NAME/KEY: 604
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: primer in a polymerase chain reaction

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CAGAGGTGCC ACAAAGCTGG

20

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Unkown
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Oligonucleotides

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## (ix) FEATURE:

- (A) NAME/KEY: 605
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: primer in a polymerase chain reaction

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCAGCTTTGT GGCACCTCTG

20

## (2) INFORMATION FOR SEQ ID NO:16:

10

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Unknown
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Oligonucleotide

15

## (ix) FEATURE:

- (A) NAME/KEY: 606
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: primer in a polymerase chain reaction

20

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CATCATGGGG ACCCTCACGG

20

## (2) INFORMATION FOR SEQ ID NO:17:

25

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Unknown
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Oligonucleotide

30

## (ix) FEATURE:

- (A) NAME/KEY: 2213
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: primer in a polymerase chain reaction

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

35

AGGATGCAGG CCCTGGTGCT

20

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## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Unknown
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Oligonucleotide

## (ix) FEATURE:

- (A) NAME/KEY: 2744
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: primer in a polymerase chain reaction

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCTCCTCCAC CAGCGCCCCT

20

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Unknown
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Oligonucleotide

## (ix) FEATURE:

- (A) NAME/KEY: 2238
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: primer in a polymerase chain reaction

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

ATGATGTCGG ACCCTAAGGC TGTT

24

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Unknown
- (D) TOPOLOGY: Unknown

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(ii) MOLECULE TYPE: Oligonucleotide

(ix) FEATURE:

(A) NAME/KEY: 354

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: primer in a polymerase chain reaction

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TGGGGACAGT GAGGACCGCC

20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Oligonucleotide

(ix) FEATURE:

(A) NAME/KEY: JT10 - UP01

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: primer in a polymerase chain reaction

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGTGTGCAAA TGTGTGCGCC TTAG

24

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Oligonucleotide

(ix) FEATURE:

(A) NAME/KEY: JT10 - DP01

(B) LOCATION:

(C) IDENTIFICATION METHOD:

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(D) OTHER INFORMATION: primer in a polymerase chain reaction

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGGAGCTGCT TTACCTGTGG ATAC

24

5

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

10

(ii) MOLECULE TYPE: Oligonucleotide

(ix) FEATURE:

(A) NAME/KEY: 1590

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: primer in a polymerase chain reaction

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGACGCTGGA TTAGAAGGCA GCAAA

25

20

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

25

(ii) MOLECULE TYPE: Oligonucleotide

(ix) FEATURE:

(A) NAME/KEY: 1591

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: primer in a polymerase chain reaction

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCACACCCAG CCTAGTCCC

19

35

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## (2) INFORMATION FOR SEQ ID NO:25:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Genomic DNA

## (ix) FEATURE:

- (A) NAME/KEY: 5' splice site of EXON 1
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 5' Splice Donor site is located between nucleotides 9 and 10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TATCCACAGG TAAAGTAG

18

## (2) INFORMATION FOR SEQ ID NO:26:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Genomic DNA

## (ix) FEATURE:

- (A) NAME/KEY: 5' splice site of EXON 2
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 5' Splice Donor site is located between nucleotides 9 and 10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCGGAGGAGG TCAGTAGG

18

## (2) INFORMATION FOR SEQ ID NO:27:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

35

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(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: 5' splice site of EXON 3

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: 5' Splice Donor site  
is located between nucleotides 9 and 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCTCGCTGGG TGAGTGCT

18

10 (2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Unknown

15 (ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: 5' splice site of EXON 4

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: 5' Splice Donor site is  
located between nucleotides 9 and 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTGAGAAGAG TGAGTCGC

18

25 (2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Unknown

30 (ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: 5' splice site of EXON 5

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: 5' Splice Donor site is  
located between nucleotides 9 and 10

35



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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ACTTCAAGGG TGAGCGCG

18

5

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Unknown

10

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: 5' splice site of EXON 6

(B) LOCATION:

(C) IDENTIFICATION METHOD:

15

(D) OTHER INFORMATION: 5' Splice Donor site is located between nucleotides 9 and 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AGCTGCAAGG TCTGTGGG

18

20

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Unknown

25

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: 5' splice site of EXON 7

(B) LOCATION:

(C) IDENTIFICATION METHOD:

30

(D) OTHER INFORMATION: 5' Splice Donor site is located between nucleotides 9 and 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

AGGAGATGAG TATGTCTG

18

35

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## (2) INFORMATION FOR SEQ ID NO:32:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Genomic DNA

## (ix) FEATURE:

- (A) NAME/KEY: 5' splice site of EXON 8
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 5' Splice Donor site is located between nucleotides 9 and 10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TTTATCCCTA ACTTCTGT

18

## (2) INFORMATION FOR SEQ ID NO:33:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

## (ii) MOLECULE TYPE: Genomic DNA

## (ix) FEATURE:

- (A) NAME/KEY: 3' splice site of INTRON 1
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 3' Splice Acceptor site is located between nucleotides 9 and 10

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGACGCTGG

9

## (2) INFORMATION FOR SEQ ID NO:34:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

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(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: 3' splice site of INTRON 2

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: 3' Splice Acceptor site  
is located between nucleotides 9 and 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TTCTTGCAGG CCCCAGGA

18

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: 3' splice site of INTRON 3

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: 3' Splice Acceptor site  
is located between nucleotides 9 and 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TCCTGCCAGG GCTCCCCA

18

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 Base Pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

(A) NAME/KEY: 3' splice site of INTRON 4

(B) LOCATION:

(C) IDENTIFICATION METHOD:

(D) OTHER INFORMATION: 3' Splice Acceptor site  
is located between nucleotides 9 and 10

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CTCTGGCAGG AGCGGACG

18

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

- (A) NAME/KEY: 3' splice site of INTRON 5
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 3' Splice Acceptor site is located between nucleotides 9 and 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TCTTCTCAGA GCTGCGCA

18

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Genomic DNA

(ix) FEATURE:

- (A) NAME/KEY: 3' splice site of INTRON 6
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: 3' Splice Acceptor site is located between nucleotides 9 and 10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TCTTTCCAGG GCAGTGGG

18

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

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- 5 (ii) MOLECULE TYPE: Genomic DNA  
(ix) FEATURE:  
    (A) NAME/KEY: 3' splice site of INTRON 7  
    (B) LOCATION:  
    (C) IDENTIFICATION METHOD:  
    (D) OTHER INFORMATION: 3' Splice Acceptor site  
        is located between nucleotides 9 and 10
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:  
TTGTCTCAGA TTGCCCAG 18
- 10 (2) INFORMATION FOR SEQ ID NO:40:
- 15 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 18 Base Pairs  
    (B) TYPE: Nucleic Acid  
    (C) STRANDEDNESS: Double  
    (D) TOPOLOGY: Unknown
- 20 (ii) MOLECULE TYPE: Genomic DNA  
(ix) FEATURE:  
    (A) NAME/KEY: 3' splice site of INTRON 8  
    (B) LOCATION:  
    (C) IDENTIFICATION METHOD:  
    (D) OTHER INFORMATION: 3' Splice Acceptor site  
        is located between nucleotides 9 and 10
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:  
TCTCTACAGA GCTGCAAT 18
- 25 (2) INFORMATION FOR SEQ ID NO:41:
- 30 (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 737 Base Pairs  
    (B) TYPE: Nucleic Acid  
    (C) STRANDEDNESS: Double  
    (D) TOPOLOGY: Unknown
- 35 (ii) MOLECULE TYPE: Genomic DNA  
(ix) FEATURE:  
    (A) NAME/KEY: PEDF Promoter  
    (B) LOCATION:  
    (C) IDENTIFICATION METHOD:  
    (D) OTHER INFORMATION: EXON begins at 614 and  
        ends at 728 of PEDF GENE

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

	TTCTTTTTTTT GAGACGGGGT CTCGCTCTGC TCGCCCAGGA	40
5	TGGAGTGCAG TGGTGTGATC TCAGCTCACT GCAACCTCCG	80
	CCTCCCAGGT TTAAGTGATT CTCCTGCCTC AGACTCCCAA	120
	GTAGCTGGGA CTACAGGTGC GCGCCAACAC ACCTGGGTAA	160
	TTTTGTTTGT ATTTT TAGTA GAGATGGGGT TTCACCGTGT	200
10	TGACTAGGCT GGTCTCGAAC CTCCTGACCT CAGGTGATCC	240
	CCCGGCCTCG GTCTCCCAA GTGCTGGGGA TAACAAGCGT	280
	GAGCCACTGC GCCCAGCTTT GTTTGCATTT TTAGGTGAGA	320
	TGGGGTTTCA CCACGTTGGC CAGGCTGGTC TTGAACTCCT	360
15	GACCTCAGGT GATGCACCTG CCTCAGTCTC CCAAAGTGCT	400
	GGATTACAGG CGTTAGCCCC TCGCCCCGGC CCCTGAAGGA	440
	AAATCTAAAG GAAGAGGAAG GTGTGCAAAT GTGTGCGCCT	480
	TAGGCGTAAT GGATGGTGGT GCAGCAGTGG GTTAAAGTTA	520
20	ACACGAGACA GTGATGCAAT CACAGGAATC CAAATTGAGT	560
	GCAGGTCGCT TTAAGAAAGG AGTAGCTGTA ATCTGAAGCC	600
	ATCTGAAGCC TGCTGGACGC TGGATTAGAA GGCAGCAAAA	640
	AAAGCTCTGT GCTGGCTGGA GCCCCCTCAG TGCAGGCTTA	680
25	GAGGGACTAG GCTGGGTGTG GAGCTGCAGC GTATCCACAG	720
	GCCCCAGGGT AAAGTAG	737

## (2) INFORMATION FOR SEQ ID NO:42:

30

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 88 Base Pairs
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Double
  - (D) TOPOLOGY: Unknown

35

- (ii) MOLECULE TYPE: Genomic DNA

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## (ix) FEATURE:

- (A) NAME/KEY: PEDF Promoter
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: EXON PEDF GENE  
begins at 9

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TTCTTGCAGA TGCAGGCCCT GGTGCTACTC CTCTGCATTG	40
GAGCCCTCCT CGGGCACAGC AGCTGCCAGA ACCCTGCCAG	80
CCCCCCGG	88

10

## (2) INFORMATION FOR SEQ ID NO:43:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22481 Base Pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Double
- (D) TOPOLOGY: Unknown

15

## (ii) MOLECULE TYPE: Genomic DNA

## (ix) FEATURE:

- (A) NAME/KEY: P1-147
- (B) LOCATION:
- (C) IDENTIFICATION METHOD:
- (D) OTHER INFORMATION: full length genomic  
sequence for PEDF plus flanking sequences.

20

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCGGCCGCAG GGTGGACTGT GCTGAGGAAC CCTGGGCCCA	40
GCAGGGGTGG CAGCCCGCGC AGTGCCACGT TTGGCCTCTG	80
CCCGCTCGCC AGGCATCCTC CACCCCGTGG TCCCCTCTGA	120
CCTCGCCAGC CCTCCCCCGG GACACCTCCA CGCCAGCCTG	160
GCTCTGCTCC TGGCTTCTTC TTCTCTCTAT GCCTCAGGCA	200
GCCGGCAACA GGGCGGCTCA GAACAGCGCC AGCCTCCTGG	240
TTTGGGAGAA GAACTGGCAA TTAGGGAGTT TGTGGAGCTT	280
CTAATTACAC ACCAGCCCCT CTGCCAGGAG CTGGTGCCCCG	320
CCAGCCGGGG GCAGGCTGCC GGGAGTACCC AGCTCCAGCT	360
GGAGACAGTC AGTGCCTGAG GATTTGGGGG AAGCAGGTGG	400

35

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	GGAAACCTTG GCACAGGGCT GACACCTTCC TCTGTGCCAG	440
	AGCCCAGGAG CTGGGGCAGC GTGGGTGACC ATGTGGGTGG	480
5	GCACGCTTCC CTGCTGGGGG TGCAGGGGGT CCACGTGGCA	520
	GCGGCCACCT GGAGCCCTAA TGTGCAGCGG TTAAGAGCAA	560
	GCCCCCTGGAA GTCAGAGAGG CCTGGCATGG AGTCTTGCTT	600
	CTTGCAAACG AGCCGTGTGG AGAGAGAGAT AGTAAATCAA	640
10	CAAAGGGAAA TACATGGTCT GTCCGAGGAT GAGCTGCCGG	680
	AGAGCAATGG TGAAAGTGAA GTGGGGGAGG GGGCGGGGCT	720
	GGGAGGAAAA GCCTTGTGAG AAGGTGACAC GAGAGCACGG	760
	CCTTGAAGGG GAAGAAGGAG GGCATATGG AGGTCCCGGC	800
15	GAAGCGTGGC CTGGCCGAGG AACGGCATGT GCAGAGGTCC	840
	TGCCGAGGAG CTCAAGACAA GTAGGGGACG GTGGGGCTGG	880
	AGTGGAGAGA GTGAGTGGGA GGAGGAGTAG GAGTCAGAGA	920
	GGAGCTCAGG ACAGATCCTT TAGGCTCTAG GGACACGATA	960
20	AACACAGTGT TTTTGTCTT GTCAAGTGTG TCCTTTTAT	1000
	TTTTTTGAAA GAGTCTCGCT CTGTAGCCCA GGCTGGAGTG	1040
	CAGCGGTGCG ACCTCGGCTC ACTGCAACCT CTGCCTCCCG	1080
25	GGTCCAAGCA ATTCTCCTGC CTCAGCCTCC CGAGTAGCTG	1120
	GGATTACAGG CACCCGCCAC CACGCACTGC TAATTTTGT	1160
	ATTTTAGTAG AGACCGGGTT TTGCCATGTT GGTCAGGCTG	1200
	GTCTCGAACT CCTGACCTCA GGTGATCCGC CCGCCTCGGC	1240
30	CTCCCAGAGT GGTGTGAGCC ACTATGCCCT GCAGCACTTG	1280
	TCAAGTCTTT CTCAGCGTTC CCCTCCTCTC CACTGCAGCT	1320
	CCCAGTGCCC CAGTCTGGGC CTCGTCTTCA CTTCTGGGA	1360
	TCCCTGACAT TGCCTGCTAG GCTCTCCCTG TCTCTGGTCT	1400
35	GGCTGCCTTC ACTGTAACCT CCACCCAGCA GGTACCTCTT	1440



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	CAGCACCTCC	CATGAACCCA	GCAGAATACC	AAGCCCTGGG	1480
	GATGCAGCAA	CGAACAGGTA	GACGCTGCAC	TCCAGCCTGG	1520
5	GCGACAGAGC	AAGACTCCGC	CTGAAGAAAA	AAAAAAGGAC	1560
	CAGGCCGGGC	GCGGTGGCTC	ACGCCTGTAA	TCCCAGCACT	1600
	TTGGGAGGCC	GAGGTGGGTG	GATCATGAGG	TCAGGAGTTC	1640
	AAGACCAGCC	TGGCCAAAAT	GGTGAAACCC	CGTCTCTACT	1680
10	GAAAAATACA	AAAATTAGCT	GGGTGCAGTG	GCGGGCGCCT	1720
	GTAGTCTCAG	CTACTCAGGA	GGCTGAGGCA	GGATAATTGC	1760
	TTGACCCCAG	GAGGCAGAGG	TTGCAGTGAA	CCGAGATCAC	1800
	GCCACTGCAC	TCCAGCCTGG	GCGACAGAGC	AAGACTCTGC	1840
15	CTCAAAAAAA	AGAATAAAAA	TAAAAAAAAG	GACCAGATAC	1880
	AGAAAACAGA	AGGAGACGTA	CTATGAAGGA	AATTGGAGAG	1920
	CTTTTGGGAT	ACTGAGTAAC	TCAGGGTGGC	CTTTCCCAGG	1960
	GGACATTTAG	CTGAGAGATA	GACGGTATGA	AGACCTGACC	2000
20	G TTCAGAAAC	AGGGGAAGAG	GCAGCAGCCC	GGGCAAAGGC	2040
	CTTTGGGGCA	GGAAAGGGCT	TGGATCACTG	GAGAAGCAGA	2080
	AAGATGGCCA	GTGTGACCAG	AGTGTGACAA	AGTCAGAGAA	2120
25	AACCAGGAAG	ATGGAGCTGG	AGACACAGGC	GGGGCCAGAT	2160
	CACGAGGGTC	CTCGCAGACC	AGAGCAAGGG	TTTGGATTTT	2200
	ATTCCAAGTA	TGAAGGGAAG	CTGCTGAAGT	GTGTTTTCTT	2240
	TTACAATTTG	TAGTTGAAAT	ATAATATGCA	AAGTACACAA	2280
30	GTCTTAACTA	TATGTAAGCT	TAATGAATGT	TTCCATGAAC	2320
	CAAATACCGC	TGTGCAACCA	TCACCAGCTC	AAGAGACGAA	2360
	CCCTTCTCCC	TCCTCCTGAC	TGCCAGTAAC	ATAGTGGTTC	2400
	AGCTCAAGAA	ACAGAACTCT	TCTGACTTCC	CCTAACATAG	2440
35	CGGGTTTTCT	TTTTTGTTTT	GTTTTTTGTT	GTTTTTTAAG	2480

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	AGACAATGTC	TTTATTATTT	TTATTTTTTTT	TTATTTTTTGA	2520
	GACGGAGTCT	TGCTGTCGCC	CAGGCTGGAG	TGCAGTGGTG	2560
5	CGATCTCGGC	TCACTGCAGG	CTCTGCCCCC	CGGGGTTCAT	2600
	GCCATTCTCC	TGCCTCAGCC	TCCCTAGCAG	CTGGGACTAC	2640
	AGGTGCCCCG	CACCTCGCCC	GGCTATTTTTT	TTGTATTTTTT	2680
	AGTGGAGACG	GGGTTTCACC	GTGTTAGCCA	GGATGGTCTC	2720
10	GATCTCCTGA	CCTCGTGATC	CGCCACCTC	GGCCTCCCAA	2760
	AGTGCTGGGA	TTACAGGCAT	GAGCCACCGC	GCCCAGCCAA	2800
	GAGACACGGT	CTTGCTCTGT	CGCCCAGGCT	GGATGGAGTG	2840
	CCGTGGTGCG	ATCACAGCTC	GCGGCAGCCT	TGACATCCTG	2880
15	GGCTCAAGCA	ACCTTCCTGC	CTTGGCCTCC	CAAATGTTGG	2920
	GATTATAGGC	ATGAGCCACT	GTGCTTGGA	TCTATTCATC	2960
	TTTAATGTCA	AGCAGGCAAT	TGAATATTTG	ATCAGGGATA	3000
	GAATTGTCTA	TTTGGGGGTA	TGCAGATGTG	CTTCATGTCA	3040
20	TGGAAC TGGG	CCGGGCGCGG	TGGCTCATGC	CTATAATCCC	3080
	AGCACTTTGG	GAGGCCGAGG	CAGGCGGATC	ATAAGGTCAG	3120
	GAGATCGAGA	CCATCCGGGC	CAACACGGTG	AAACCCCGTC	3160
25	TCTACTAAAA	ATACAAAAAT	TAGGCAGGTG	TGGTGGTGCG	3200
	TGCCTGTAGT	CCCAGCTACT	CAGGGAGGCT	GAGACAGGAG	3240
	AATTGATTGA	ACCTGGGAGG	CAGAGGTTGT	AGTGAGCCAA	3280
	GATCGCGCCA	CTGCACTCCA	GCCTGGGCGA	CATGAGCGAG	3320
30	ACTCCGTCTC	AAAAATAAAC	AAAAAAAAGT	CATGGAATTG	3360
	ATGGAAATTG	CCTAAGGGGA	GATGTAGAAG	AAAAGGGGTC	3400
	TCAGGATCAA	GCCAGCAGAG	AAGGCAGAAA	AGGTAAGGTG	3440
	TGTGAGGTGG	CAGAAAAAGG	GAAGAGTGTG	GACAGTGAGG	3480
35	GTTTCAAGGA	GGAGGAACTG	TCTACTGCCT	CCTGCCAAGG	3520

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	ACGGAGGTGT	CCACTGCCAG	TTGACATAAG	GTCACCCATG	3560
	AACTTGGTGA	CAGGAATTTC	AGTGGAGAAG	TGGCCACAGA	3600
5	CACAAGTCTA	GAATTGAAAT	GGGAGCCGAG	GCAGCGTAGA	3640
	CAAAAGAGGA	AACTGCTCCT	TCCAGAGCGG	CTCTGAGCGA	3680
	GCACCGAGAA	ATGGGCAGTG	GCTTTAGGGG	ATGTAGCGTC	3720
	AAGGAAGTGT	CTTTTAAAGA	AGTCGGGGGC	CGGGCACGGT	3760
10	GGCTCACGCC	TGTAGTCCCA	GCACTTTGGG	AGGCCGAGGC	3800
	AGGCAGATCA	CTTGAGGTCA	GGAGTTCGAG	ACCAGCCTGG	3840
	CTAACACGAT	GAAACCCCGT	CTCTACTAAA	AATACAAAAA	3880
	ATTAGCTGGG	CACGGTGGCT	CGTGCCTGTA	ATCCCAGCAC	3920
15	TTTGGGAGGC	AGAGGTGGGC	AGATCACTTG	AGGTCAGGAG	3960
	TTTGAGACCA	GCCTAGCCAA	CATGGTGAAA	CCCCATCTCT	4000
	ACTAAACTA	CAAAAATTAG	CCGGGAGTGG	TGGCACGTGC	4040
	CTGTAATCCC	AGCCAGTCAG	GAGGCTGAGG	CAGGAGAATC	4080
20	ACTGGAATCC	TGGAGGTGGA	GGTGGCAGTG	AGCCGAGATG	4120
	GTACCTCTGT	ACTCCAGCCT	GGGGGACAGA	GTGAGACTCC	4160
	GTCTCAAAAA	AAAAAGAAGG	TGGGGAAGGA	TCTTTGAGGG	4200
	CCGGACACGC	TGACCCTGCA	GGAGAGGACA	CATTCTTCTA	4240
25	ACAGGGGTCG	GACAAAAGAG	AACTCTTCTG	TATAATTTAT	4280
	GATTTTAAGA	TTTTTTATTTA	TTATTATTTT	TTATAGAGGC	4320
	AAGCATTTTT	CACCACGTCA	CCCAGGCTGG	TCTCCAACTC	4360
	CTGGGCTCAA	GTGTGCTGGG	ATTATAGCCA	TGAGTCACCA	4400
30	CACCTGGCCC	AGAAACTTTA	CTAAGGACTT	ATTTAAATGA	4440
	TTTGCTTATT	TGTGAATAGG	TATTTTGTTT	ACGTGGTTCA	4480
	CAACTCAAAA	GCAACAAAAA	GCACCCAGTG	AAAAGCCTTC	4520
35	CTCTCATTCT	GATTTCCAGT	CACTGGATTC	TACTCTTGGG	4560

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	ATGCAGTGTT TTTCATCTCT TTTTGTATC CTTTGGAAA	4600
	TAGTATTCTG CTTTAAAAAG CAAATACAGG CCAGGTATGG	4640
5	TGGCTCACTC CTGTAATCCC AGCACTTTGG GAGGCCGAGG	4680
	CAGGTGATCA CCTAAGGTCA GGAGTTCAAG ACCAGCCTGG	4720
	CCAATATGGT GAAACCCTGT CTGTACCAA ACACAAAAAC	4760
	AAAAACAAA ACAAAAATTA GCCGGGCGTG GTGGCGTGCT	4800
10	CCTGTAATCC CAGCTACTCA GGAGGCTGAG GCAGGAGAAT	4840
	CGCTTGAACC TGGGAGGCAG AGGTTGCAGT GAGCCGAGAT	4880
	TGTGCCACTG TACTCCAGCC TGGGCCACAG AGCAAGGTTT	4920
	CATCTCAAAC AAAACAAAAC AAAACAAACA AAAAAACAAA	4960
15	ACAAAAGCTA ATACAAACAC ATATACAATA GACAAAACCTG	5000
	TAAATATTTT ATTATTTTTA TTTTTTTGTAG TAGAGACAGG	5040
	GTTTCACCAT GTTGGCCAGG ATGGTCTCAA ACTCCTGACC	5080
	TCAGGTGATC CACCCACCTC AGCCTCCCGA TAGTTAGGAT	5120
20	TACAGGCATG AGCCACCACA CCCGGCCTAA AATTGTAAAC	5160
	GTTTTAGAAG AAAGTATAGA TGAATCCCTT CGTGATCTCG	5200
	GGGAAGAAGA GATTTTTTTAA AAAAGATACC AAAAGAAGCA	5240
25	CAAATTATAA AAGAAAAGAT TGAAAATGTT GGTGTTAAAA	5280
	TTAAAAACTT GTTTTAAAAC AAGCTTGTGT AACCCATGAC	5320
	CCACAGGCTG CATGTGGCCC AGAAAAGCTT TGAATGCAGC	5360
	CCAACACAAA TTCGTAAACT TTCCTAAAAC ATTATGAGAT	5400
30	TTTTTTTGAG ATTTTGTTTT GTTTTGTTTT TTGTTTTTTT	5440
	AGCTCATTCG GTATCATTAA TGTTAGCATA TTTTACGTGG	5480
	GGCCCAAGAC AATTCTTCTT CCAATGTGTC TCAGGGGAGC	5520
	CAAAAGATTG GACACCCCTG CCATAAACAT GAAAAGACAA	5560
35	TGGCCGGGCA CGGTGGCTCA CGCCTGTAAT CCCAGCACTT	5600

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	TGGGAGGCTG	AGGGGGGCGG	GATCACCTGA	GGTCAGGAGT	5640
	TTGAGACAAG	CGTGACCAAT	GTGGTGAAAC	CCTGTCTCTA	5680
5	CTAAAAATAC	AAAAATTAGC	CGGGCATGCT	CGTGCACACC	5720
	TATAGTCCCA	ACTACTCAGC	AGGGTGAGGC	AGGAGAACCT	5760
	CTTGAACCCG	GGAAGCGGAG	GTTGCAGTGA	GCCGACATTG	5800
	CACCCCTGCA	CTCCAGCCTG	GGTGACAGAG	TGAGTCTCCA	5840
10	CTGGAAAAAA	AAAAAAAAGA	ACAGTGTGAT	ACATTGACCT	5880
	AAGGTTTAAG	AACATGCAAA	CTGATACTAT	ATATCACTTA	5920
	GGGACAAAAA	CTTACATGGT	AAAAGTAAAA	AGAAATGTAC	5960
	GAAAATAATA	AAAATCAAAT	TCAAGATGGT	GGTTATGGTG	6000
15	ACGGGAAAGA	ACTGAGGCGG	AAATATAAGG	TTGTCACTAT	6040
	ATTGAGAAAT	TTTTCTATCT	TTTTTTCTTT	TTTCTTTTTT	6080
	TGAGACGGGG	TCTCGCTCTG	TCGCCCAGGA	TGGAGTGCAG	6120
	TGGTGTGATC	TCAGCTCACT	GCAACCTCCG	CCTCCCAGGT	6160
20	TTAAGTGATT	CTCCTGCCTC	AGACTCCCAA	GTAGCTGGGA	6200
	CTACAGGTGC	GCGCCAACAC	ACCTGGGTAA	TTTTGTTTGT	6240
	ATTTTTTAGTA	GAGATGGGGT	TTCACCGTGT	TGACTAGGCT	6280
25	GGTCTCGAAC	TCCTGACCTC	AGGTGATCCC	CCGGCCTCGG	6320
	TCTCCCAAAG	TGCTGGGATA	ACAAGCGTGA	GCCACTGCGC	6360
	CCAGCTTTGT	TTGCATTTTT	AGGTGAGATG	GGGTTTCACC	6400
	ACGTTGGCCA	GGCTGGTCTT	GAACCTCTGA	CCTCAGGTGA	6440
30	TGCACCTGCC	TCAGTCTCCC	AAAGTGCTGG	ATTACAGGCG	6480
	TTAGCCCCTG	CGCCCGGCCC	CTGAAGGAAA	ATCTAAAGGA	6520
	AGAGGAAGGT	GTGCAAATGT	GTGCGCCTTA	GGCGTAATGG	6560
	ATGGTGGTGC	AGCAGTGGGT	TAAAGTTAAC	ACGAGACAGT	6600
35	GATGCAATCA	CAGAATCCAA	ATTGAGTGCA	GGTCGCTTTA	6640

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	AGAAAGGAGT AGCTGTAATC TGAAGCCTGC TGGACGCTGG	6680
	ATTAGAAGGC AGCAAAAAAA GCTCTGTGCT GGCTGGAGCC	6720
5	CCCTCAGTGT GCAGGCTTAG AGGGACTAGG CTGGGTGTGG	6760
	AGCTGCAGCG TATCCACAGG TAAAGCAGCT CCCTGGCTGC	6800
	TCTGATGCCA GGGACGGCGG GAGAGGCTCC CCTGGGCTGG	6840
	GGGGACAGGG GAGAGGCAGG GGCACCTCCAG GGAGCAGAAA	6880
10	AGAGGGGTGC AAGGGAGAGG AAATGCGGAG ACAGCAGCCC	6920
	CTGCAATTTG GGCAAAGGG TGAGTGGATG AGAGAGGGCA	6960
	GAGGGAGCTG GGGGGACAAG GCCGAAGGCC AGGACCCAGT	7000
	GATCCCCAAA TCCCACTGCA CCGACGGAAG AGGCTGGAAA	7040
15	GGCTTTTGA TGAAGTGAGT GGGAAACAGC GGAGGGGCGG	7080
	TCATGGGGAG GAAAGGGGAG CTAAGCTGCT GGGTCGGGTC	7120
	TGAGCAGCAC CCCAAGACTG GAGCCCGAGG CAAGGAGGCT	7160
	CACGGGAGCT GCTTCCACCA AGGGCAGTCA GGAAGGCGGC	7200
20	CGCCCTGCAG CCCAGCCCTG GCCCCTGCTC CCTCGGCTCC	7240
	CTGCTACTTT TTCAAAATCA GCTGGTGCTG ACTGTTAAGG	7280
	CAATTTCCCA GCACCACCAA ACCGCTGGCC TCGGCGCCCT	7320
25	GGCTGAGGGC TGGGATGGAG GACAGCTGGG TCCTTCTAGC	7360
	CAGCCCCCAC CCACTCTCTT TGGCTACATG AGTCAAGGCT	7400
	GGGCGACCAA TGAGGTTGTG GCCTCCGGCA AACAATGACC	7440
	ACTATTTAGG CCGGCAGGTG TATAGGGCGT GGGGGCCCAG	7480
30	CTGCCAGTGC TGGAGACAAG GGCTGTCCGA GATGAACCCT	7520
	TTCTGCTGCC TGCCAAGCCA CTGGGAGGGG TAGGTCTCAG	7560
	CAGGATTCCC AGAAACCCCG CCCCTGTCCA GCCTAGGCCC	7600
	CCCACCCGGT GTTAGCTAAC CCAACGTTAG CCCCAGGTT	7640
35	CCGTGGGGTT GGGGGGCAGG GAGTCCTATT CTTGGGGCTG	7680

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	CTGCTTCTGG	GGTGTGGGGA	AGTGCAACTC	CACGGCACCC	7720
	TGGGCTGACT	CATTCAGCTT	CTAAAGCTTC	AGGAAACATT	7760
5	GTTTGGGGCT	GGGTCACCAT	GGGTGGGCCA	GAGAGGACCC	7800
	CTCAATCCCC	TCCGGAGAGC	CAGGGGAGGG	GGAGGTGCCC	7840
	TTCCCCATGC	TATCTCCGAG	GCCCACTGCC	ATGTGGCTGA	7880
	AGGCTGTGCG	GTTCTGGGAA	GAGGGGGAGG	TGGCGGTGGA	7920
10	GGCTGTTTGT	CTCCTAACTG	GGCTTAATCT	GAAACACATG	7960
	TATTGGCTTG	AGTTGATCCG	CCTCACGTGG	AGGCAAGATC	8000
	ACAAAAGCTT	CTGTGTTTCT	TGATGTGGGC	AATTGTCAGA	8040
	AAATAAGGCC	TGACCTTGGC	CCAGCAGGGA	GGGTATCTAC	8080
15	CTCTCCCTGA	GCCCTCCCCC	GCCTGCTAGG	ACGAGAGCGG	8120
	GGCTTGGATA	CTGCCCTTTG	GACAGGATGG	CATCATTGTC	8160
	TGTGGCTGCA	GCCAGCCAGC	GGTCGCCTGC	TCAGCCCATG	8200
	AGCAACCACT	GTGGACAGGG	TATTGCGTGT	GTGCTGAGGG	8240
20	GCGTCCATGC	AGACCCCCAC	GCTTGCCCTC	TCACTGCCCT	8280
	TGTAGGGTTT	TCAATCATCT	CTCCTCTTCC	CTTATCCAGA	8320
	TGGCTTGAAG	TGGAGGATTC	AGACTTGCCG	TTAATACTCT	8360
	GGGTCCCTGT	GTCTAGCTCG	GGGCCACCTT	TGGACCCATG	8400
25	TCCCTTCCCT	GCCAGGCTCC	CTCACCTCAC	CTCAGCCTAC	8440
	CCACATTGTG	ACAATCATCT	ACCACCTGAT	CTGGGGTTTG	8480
	GGCTTAGATT	CTGTAGGCAC	CAAGACTAAA	GTCGCTCCTT	8520
30	CAAGTCCATT	TGAATTGTGA	CTTTAGTTTC	CTTAAATACT	8560
	ATGCCAGGAT	AATGGCCAGG	GATGGTGGCT	CACGCCTGTA	8600
	CTCCTGGCAC	TTTGGGATGC	TGGTGGATCA	CCTGAGATCA	8640
	GGATTCCAGG	CCAGCCTGGC	CAACACGGTG	AAACCCCATC	8680
35	TCTACTAAAA	CATAAAAATT	AACCAGGTGT	GGTGGCGGGC	8720

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	ACCTGTAATC CCAGCTACTC AGGAGACTGA GGCAGGAGAA	8760
	TTGCTTGAAC CCGGGAGGTG GAAGTTGCAC TGAGCTGAGA	8800
5	TCGCGCCACT GCACTTTAGC CTGGGCGACA AGAGTGAAAC	8840
	TCTGTCTCAA AAACAAAAAA AACTATGCCG GGATGAGCCT	8880
	GTCTCCTCCC TTAATTTCTT ACTTGGGCCA GAGGAAGTAG	8920
	AACTAACAAC TTCTCTTCTA GCCTTGCCTC CTGTGTACCT	8960
10	CACTGAATTT TTGGTCTCTA ATAAACCAGT CTGCAGAGGC	9000
	TCAGGGGAGG CAGGCTCCTG GCAGCTGGGT GGGGCTGGCC	9040
	CCAGCCGGGT GGAGACCAGC TGTAGGCCTG GATGGTGGTG	9080
	AGGCCTCTGT CTTGCACTGC AGAAAGCTTT TCCTGTTGTC	9120
15	TACACGAAAG TTTTCTCCCT GCATGTCAGG GCAGCCACGT	9160
	GCAAGAGCAG CTGGCTGGGA ACGCAGAGGT CTGCGGCTCG	9200
	AGGCGGGGTT TAGAAAGAAA ACCAGGCTGC TTCCTGCTGC	9240
	CCGTCTTGCC TTAAGCTGAG TAAACTCAAA GGCAATCTTC	9280
20	TTTCATGCCT CACGATATTG TCCAGTGGAT TATCTGATTT	9320
	AATTTGAAGG ACGAGAGCCA ACAATCACAC AACGTCCTCC	9360
	CAAATTTTCT GATCCACTTT GTTCTGGGAA GTCAAAAAGT	9400
25	GCGTGTGCTG TGTGGGTGGA TGTTTGTGTA TATAAATGGA	9440
	TAATGAAGGA TGATGTGTTG GGGGCCAGGG CAGGGGAGAC	9480
	AACGCTGTTC AGATTCTACA TTTTTTTTTT CTTTTTTTTT	9520
	TTTTTTTGTAG ATGGAGTCTT GCTCTGTTGC CCAGCCTGGA	9560
30	GTGCAGTGGC GCGATCTCAG CTCACTGCAA CCTCCACTTC	9600
	CTGGATTCAA GTGATTCTCC TGCCTTAGCC TCCCAAGTAG	9640
	CTGGGATTAC AGGCATGCGC CACCACACCC GGCTAATTTT	9680
	TGTATTTTTTA GTAGAGATGG GGTTTCTCCA TGTTGGCCAG	9720
35	GATGGTCTCA AACTCCTGAC CTCAGGTGAT CTACCCGCCT	9760



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	CGGCCTCTCA AAGTGCTGGG ATTACAGGTT TGAGCCACTG	9800
	CGCCTGGCCT TTTTTTTTTT TTTTGAGATG GAGTTTTTCAC	9840
5	TCTTGTTGCC CAGGCTGGAG TGCAGTGGTG CGATCTTGGC	9880
	TCACTGCAAC CTCCACCTCC CAAGTTCAAG TGATTCTCCA	9920
	GCCTTAGCCC TCCAAGTAGC TGGGACTACA GGTGTGTGCC	9960
	ACCATGCCTG GCTATTTTAT TTTATTTTAT TTTATTTATT	10000
10	TATTTTTTGAG ACTAAGTCTT GCTCTGTTGC CCAGGCTGGA	10040
	GTGCAGTGGC ATAATCGGCT CACTGCAACC TCTGCCTCCC	10080
	AGGTTCAAGT GATTCTCCTG CCTCAGCCTC CTGAGTAACT	10120
	GGGATTACAG GGGCCTGCCA CCACGCCTGG CTACTTTTTTG	10160
15	TATTTTTTAGT ATAGATGGGG TTTCACCATG TTGGCCAGGC	10200
	TGGTCTCGAA CTCCTGACCT CAGGCTATCC GCCTGCCTCA	10240
	GCCTCCCAA AAA GTGCTGGGAT TACAGGCATG AGCCACTGTG	10280
	CTCGGTAGTT GTTTTATTTT AATAGTAGGT TATTTTATTT	10320
20	CCATTTTACA AGAGAAAAAA TGGTGATTTA AAGAGCTACT	10360
	AAGACACAGC ACTGAGACCA TGTGTGATGG CATGCGCCTG	10400
	CAGTCCCAGC TACTCACGAG GCTGAGGCAG GAGGATCACA	10440
25	TGAGGTCAGG AGTTCCAGGC TGTGGAGTGC TATGGTTGTG	10480
	TAGTGAATAG CCACTACACT CCAGCCTGGG CAGCACAGCA	10520
	AGATCTTGTC TCCCAAAAAA AAAAAAAAAA AAAAATTTCA	10560
	AATGTGAACC CAGGATCTCT GACCCTAGGC CCTGCACTCC	10600
30	TAACCATGGG AGGAAGAGCT CTTGAAAGGG AACTGTGGGA	10640
	GAAGGGAATG AGCTGCCTTG TGAGGCCACA GAAGTCCAAA	10680
	GACAGCTTGA GAATTTGGAG GGACAGCACG TGCCGGACTG	10720
	GGTGCCTCTA TGCTTGGTAT CCGGTGATTC CATGGAGGAG	10760
35	ACCTGGGTTC TGCCCCATTC TCCTGGGAGG GGTTGCCCAA	10800

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	AGTCTTATCA CCGGAGTGGG TCAGCTGCCT CCAGGACAAA	10840
	GCTTTAGCAT ACACTTGTGC TGGGCCATAC TCCACGTGGA	10880
5	GAAGCCCTGC TGGGGCTGGG GCCCCACTGC TCTGGATCTT	10920
	TAAAGCTAT TGGTTCAGGG GCCAGGTGTA ATGGCTCACA	10960
	CCTATAACCC TAGCACTTTG GGAGGCTGAA GCAGGTGGAT	11000
	AGCCTGAGGT CAGGAGTTTG AGACAAGCCT GATGAACGTG	11040
10	GTGAAACCCC ATCGCTATTA AAATACAAA AATTAGCCGG	11080
	GCATGGTGGC AGGTGCCTGT AATTCCAGCT ACTTGGGAGG	11120
	CTGAGGCGGG AGAATCGCTT GAACCCAGGA GGCGGAGGTT	11160
	GCAGTGAGCC AAGATCGCTC CACTGTACTC CAGCCTGGGC	11200
15	GACAGAGCCA GACTCTGTTT CAAAAATAA AATATAAATA	11240
	AATAAATAA TAAATAAATA AATAAATAA AGCTTTAGGC	11280
	TTAAAGGAGG GTCCCCTGAC GCAGACAGTG GAACAAAAGC	11320
	ACAAGCTTAT GGTATGACTG TGGGCCCTGA GGCAGGGGGA	11360
20	GGGGCGGGAG AACCTTGCTG GGAGGGATGG GCCATCAAGC	11400
	TGAGGGTCCA CTTCTGGGGG CCTGGAGGGG TGAGGGGTGG	11440
	TCGCTGCAGG GGGTGGGGGA AAGTGA TAG CCCTGCCCAA	11480
25	CCCCTGGGTC CTGGCTGGGG TGGCCAGGAA GGGGTAGCGG	11520
	GGCAGTGCAG TGTCGGGGGA GAGCGGCTTG CTGCCTCGTT	11560
	CTTTTCTTGC AGGCCCCAGG ATGCAGGCCC TGGTGCTACT	11600
	CCTCTGCATT GGAGCCCTCC TCGGGCACAG CAGCTGCCAG	11640
30	AACCCTGCCA GCCCCCGGA GGAGGTCAGT AGGCAGGCGG	11680
	GGAGGGCGTG GTCAGCATTC CCCGCCCCTC CTTGGCAGGC	11720
	AGCACGGGAA ACAGGACAGG GAACCCGGAC CCAGGTTCCA	11760
	GGCCAGGCTT GGGCCTTTAT TTCTCTAGGG CTGGAGTTTC	11800
35	TCCAGCAGCA AAACAGAGAG AAAATGTCTT GCCTTGCCTT	11840

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	TCAGGGGATG	GAGTAGGGAC	ATGAATAAGA	TCCCAAAGA	11880
	GTAAAAATCT	GAAGCACTTT	TAACAAGTCC	AGGGCAATTC	11920
5	TCCTGCCTCA	GCTTCCCAAG	CAGCTGGGAT	TACAGGCATG	11960
	CACCACCAAG	CCCGGCTCAT	TTTGTATTTT	TAGTAGAGAC	12000
	GGGGTTTCTC	CATGTTGGTC	AGGCTGGTCT	CGAACTCCCG	12040
	ACCTCAAGTG	ATTCTCCTGC	CTCGGCCTCC	CAAAGTGCCG	12080
10	GGATGACAGG	TGTGAGCCAC	CGCACCTGGC	CAGGATCTTT	12120
	TCTCATTACC	TTGTCTTCCT	AGTGGGGGCT	CCACTGAGCA	12160
	GGTCATGTTT	CCGGACATTT	GTTCCGATAC	TGACCAGGCT	12200
	GTGGCAGGGA	GTGAGGGTAT	GGAGTGACCT	CTCTCCTGCC	12240
15	CAGAAAGGGC	GCAGCTGGGT	TCCCAAGGCA	GATACAGGCA	12280
	CATGGAGGGA	AGCCTGGGCC	ATATGAGTGT	TATGGGGTGA	12320
	GTGTTGGCGG	AGGCCACCC	TTGAGGGACA	AGAGCAGCTG	12360
	GGCATCTTGG	CGAGAGCCCT	GGACTTTCGT	GAGGTCAGAG	12400
20	TATGAATTCT	GCGTCTCCCT	CTTCCTAGCT	TTGTGACCCT	12440
	AGACAACCCT	TACCTCAGTC	TTTGCTTCCT	TGCCTATGAA	12480
	ATGGGATAAA	AACACCCATT	CTACAGGGCC	ATGTGGCCAC	12520
25	TCATTTATTT	CTCATCTACC	AAACACCTAC	TCGACAGGGG	12560
	CTGGCAATGG	GCGGAAATAA	AAACTCAGTT	CTGCCGGGTG	12600
	CGGTGGCTCA	CACCTGTAAT	CCCAGCAGTG	TGGGAGGCGG	12640
	AGCAGGACGA	TCCCTTGAAT	CCAGGAGTTT	GAGACCAGCA	12680
30	TAGGCAACAT	AGTGAGACCC	CTGTCTCTAC	ACAAAAGCAA	12720
	AAATTACCAG	GCGTGGTGGC	AAGTGCTTGT	GGTACTACCT	12760
	ACTTGGGAAG	CTGAGGTGGG	AGGATCACTT	GAGCCCAGGA	12800
	GATTAAGACT	GCAGTGAGGG	GCCGGGCGCG	GTGGCTCACG	12840
35	CCTGTAATCC	CAGCACTTTG	GGAGGTGGAG	GTGGGTGGAT	12880

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	CACGAGGTCA	GGAGATCGAG	ACCATCCTGG	CTAACACGGT	12920
	GAAACCCCGT	CTCTACTAAA	AATACAAAAA	ATTAGCTGGG	12960
5	TGTGGTGGGG	GGCGCCTGTA	GTCCCAGCTA	CTCGGGAGGC	13000
	TGAGGCAGGA	GAATGGCGTG	AACCCGGGAG	GTGGAGGTTG	13040
	CAGTGAGCTG	AGCTCGCACC	ACTGCACTCC	AGCCTGGGCG	13080
	ACAGAGTGAG	ACTCCGTCTC	AAAAAAAAAA	AAAAAAAAAA	13120
10	GAAAGAAAGA	AAAAGTGGAGT	TCTTTTTTTT	AACTTTCTTT	13160
	TTTGTAGAGAC	AGAGTCTCAC	TCCATCACCC	ATGCTGGAGT	13200
	ACAGTGGTGC	GATCTTGGCT	CACTGCAATC	TTGGCCTCCT	13240
	GAGTTCAACC	AATTCTCATG	CCTCAGCCTC	CCAAATAGCT	13280
15	GGGACCACAG	GCACGTGCCA	CCACGCCCCAG	CTAATTTTTT	13320
	GGGTATTTTT	AGTAGAGATG	GGGCCTCACC	ATGTTGCTCA	13360
	GGTTGGTCTG	AAACTCCTGA	GCTCAAGTGA	TCCATCTTCC	13400
	TCGGCCTGCC	AAAGTGCTGG	GATTATAGGC	ATAAGCCACT	13440
20	GCACCTAGCT	CCCAATTTTT	ATATTTATAT	TTATTTTTAT	13480
	TTACTTATTT	ATTTTTTGAG	ACAGGGTCTC	ACTCTGTCAC	13520
	CCAGGCTGGA	GTACAGTGGC	ACTATCTCAG	CTCACTGCAA	13560
25	CCTCTGCCTC	CTGGGTTCAA	GCGAATCTCG	TGCCTCAGCC	13600
	TCCTGAGTAG	CTGGGATTAC	AGGCATGCAC	CACCATGCCC	13640
	CGTTAATTTT	TTTGTATTTT	TAGTAGAGAC	GGGTTTCACC	13680
	GTGTTGCCCA	GGATGGTCTC	GAACTCCTGA	CCTCAAGTGA	13720
30	TTCACCCACC	TCAGCCTCCC	AAAGTGCTGG	GATTATAGGT	13760
	GTGAGCCACT	CGGCTGATGG	TTTTTAAAAA	GTGGGTTCATG	13800
	GGGCTGGGCG	CGGTGGCTCA	TGCCTGTAAT	CCCAGCACTT	13840
	TGGTAGACCG	AGGCGGGTGG	ATCACAAGGT	CAGGAGATCG	13880
35	AGACCATCCT	GCCTAACACG	GTGAAACCCC	GTCTCTACTA	13920

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	AAAATACAAA AAATTACCCA GGCATGGTGG TGGGCGCCTG	13960
	TAGTCCCAGC TACTCGGGAG GCTGAGGCAG GAGAATGGCG	14000
5	TGAACCTGGG AGGCGGAGCT TGCAGTGAGC CGAGATCACG	14040
	CCACCGTACT CCAGCCTGAG CGACAGAGCG AGACTCCGTC	14080
	TCAAAAAAAAA AAAAAAAAAAG TGGGTCATAG GTTTCGGCTT	14120
	ATAGGTCACA AGTGTTTAAA CCTGGCCATG AGGCCAGGCG	14160
10	CAGTGGCGCA TGCCTGTAAT CCCAGCCATT TGGGAGGCTA	14200
	AGGCAGGAAA ATCGCTTGAA CCGGGGAGGT GGAGGTTGCA	14240
	GTGAGCTGAG ATCGCGCCAC TGAACCTCTAG CCTGGGTGAC	14280
	ACAGTAAGAC TCTGTCTCAA ATAAAAAAAA AAACAGCTGA	14320
15	TCTCTCTTCT GCGCTGTCTC TCCACAGAGA GTCATGCGT	14360
	GATCAGGGAG TAAAACTCAT TCCCGTTTTA GGCCAAACAC	14400
	AGAAAAATTA GGAAGGACAG CCCCAGGGG CCAGAACCAC	14440
	CACCCTACAC AAAGCCGTGA GGAGACAGTC CCTGTGCATC	14480
20	TCTGCGAGTC CCTGAACTCA AACCCAAGAC TTCCTGTCTC	14520
	CTGCCAGGGC TCCCAGACC CCGACAGCAC AGGGGCGCTG	14560
	GTGGAGGAGG AGGATCCTTT CTTCAAAGTC CCCGTGAACA	14600
25	AGCTGGCAGC GGCTGTCTCC AACTTCGGCT ATGACCTGTA	14640
	CCGGGTGCGA TCCAGCATGA GCCCCACGAC CAACGTGCTC	14680
	CTGTCTCCTC TCAGTGTGGC CACGGCCCTC TCGGCCCTCT	14720
	CGCTGGGTGA GTGCTCAGAT GCAGGAAGCC CCAGGCAGAC	14760
30	CTGGAGAGGC CCCCTGTGGC CTCTGCGTAA ACGTGGCTGA	14800
	GTTTATTGAC ATTTTCAGTTC AGCGAGGGGT GAAGTAGCAC	14840
	CAGGGGCCTG GCCTGGGGGT CCCAGCTGTG TAAGCAGGAG	14880
	CTCAGGGGCT GCACACACAC GATTCCCCAG CTCCCCGAAA	14920
35	GGGGCTGGGC ACCACTGACA TGGCGCTTGG CCTCAGGGTT	14960

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	CGCTTATTGA CACAGTGACT TCAAGGCACA TTCTTGCATT	15000
	CCTTAACCAA GCTGGTGCTA GCCTAGGTTC CTGGGATGTA	15040
5	ACTGCAAACA AGCAGGTGTG GGCTTGCCCT CACCGAGGAC	15080
	ACAGCTGGGT TCACAGGGGA ACTAATACCA GCTCACTACA	15120
	GAATAGTCTT TTTTTTTTNT TTTTTTNNC TTTCTGAGAC	15160
	GGAGTCTCGC TTTGTCNCCA AGGCTGGAGT GCAGTGGTGT	15200
10	GATCTCAGCT CACTGCAACC TCTGCCTCCC TGGTTCAAGG	15240
	AATTCTCCTG CCTCAGCCTC CAGAGTAGCT GGGATTACAG	15280
	GCACCTGCCA TCATGCCCAG CTAATTTTGT TATTTTGTAGT	15320
	AGAGACGGGG TTTCACCATG TTGCCTAGGC TGGTCTCAAA	15360
15	CTCCCGGGCT CAAGCGATCC ACCCGCCTTG GCCTCCCAA	15400
	GTGCTGGGAT TACAGGCGTG AGCCACCGCG CCTGGCCAGA	15440
	ATAATCTTAA GGGCTATGAT GGGAGAAGTA CAGGGACTGG	15480
	TACCTCTCAC TCCCTCACTC CCACCTTCCA GGCCTGATGC	15520
20	CTTTAACCTA CTTCAGGAAA ATCTCTAAGG ATGAAAATTC	15560
	CTTGGCCACC TAGATTGTCT TGAAGATCAG CCTACTTGGG	15600
	CTCTCAGCAG ACAAAAAAGA TGAGTATAGT GTCTGTGTTC	15640
	TGGGAGGGGG CTTGATTGTG GGCCCTGGTG TGCAGTTATC	15680
25	AACGTCCACA TCCTTGTCTC TGGCAGGAGC GGAGCAGCGA	15720
	ACAGAATCCA TCATTCACCG GGCTCTCTAC TATGACTTGA	15760
	TCAGCAGCCC AGACATCCAT GGTACCTATA AGGAGCTCCT	15800
30	TGACACGGTC ACTGCCCCC AGAAGAACCT CAAGAGTGCC	15840
	TCCCGGATCG TCTTTGAGAA GAGTGAGTCG CCTTTGCAGC	15880
	CCAAGTTGCC TGAGGCATGT GGGCTCCATG CTGCAGGCTG	15920
	GGGGGGTCTT TTTTTTTTTT GGGGAAAGAC GGAGTCTCGC	15960
35	TCTGTTGCCC AGGTTGGAGT GAAGTGGCGT GATCTCGGTT	16000

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	CACTGAAACC CCCACCTCCC GGGTTCACAC CATCCTCCTG	16040
	CCTCAGCCTC CCGAGTAGCT GGGACTGCAG GNGCCCAGCT	16080
5	AATCTTTNTT GTATTTTTAG CAGAGACGGG GTTTCACCGT	16120
	GTTTGCCAGG ATAGTCTCGA TCTCCTGACC TGGTGTCTG	16160
	CCCGCCTCGA CCTCCCAAAG TGCTGGGATT ACAGGTGTGA	16200
	GCCACCGCGC TCGGCCCGTT TCTAAACAAT AGATCATGTG	16240
10	TGCCCAGGCC TGGCCTGGCA CTGGTGTGGA GGAAGGGCCC	16280
	GTGAGCCCAA AGAGGCTCAG AAAGAGGAAG TGGGCTGCAG	16320
	GAGACGGTGG GAGGGGCAGG GAGGGCAGTG GCGCGATGTG	16360
	GGGAAATCTG CTGCCCCCCT GGCCAGTGCC TGGGGATGCC	16400
15	AGCAGAAGTC CTGGCAAGTC ACAGGAAGAT GCTGGCTGGG	16440
	AAGTCAGGGC CTGCTGAGCG CTAAACCAGA ACCCGAGCCT	16480
	GGCAGGCTCT CAAAGACGGG ATGCTTGTCG TCGAGTCTCA	16520
	TACGCTAACC TCTGCTCCGC CTCTTCTCAG AGCTGCGCAT	16560
20	AAAATCCAGC TTTGTGGCAC CTCTGGAAAA GTCATATGGG	16600
	ACCAGGCCCA GAGTCCTGAC GGGCAACCCT CGCTTGGAAC	16640
	TGCAAGAGAT CAACAACCTGG GTGCAGGCGC AGATGAAAGG	16680
25	GAAGCTCGCC AGGTCCACAA AGGAAATTCC CGATGAGATC	16720
	AGCATTTCTC TTCTCGGTGT GGCGCACTTC AAGGGTGAGC	16760
	GCGTCTCCAA TTCTTTTTCA TTTATTTTAC TGTATTTTAA	16800
	CTAATTAATT AATTCGATGG AGTCTTACTC TGTAGCCCTA	16840
30	ACTGGAGTGC AGTGGTGCGA TCTCAGCTCA ATGCAACCTC	16880
	CGCCTCCCAG GTTCAAGCAA TTCTTGTGCC TCAGCCTCCC	16920
	GAGTAGCTGG GATTACAGGG ATGTACCACC ACTCCCGGCT	16960
	AATTTTTTGT ATTTAATAGA CATGGGGTTT CACCATGTTG	17000
35	GCCAGGCTGG TCTCGAACTC CTGAGCTCAG GTGGTCTGCC	17040

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	CGCCTCAGCC	TCCCAAAGTG	CTAGGATTAC	AAGCTTGAGC	17080
	CACCACGCCC	AGCCCTTTTTT	ATTTTTTAAAT	TAAGAGACAA	17120
5	GGTGTTGCCA	TGATGCCCAG	GCTGGTCTCG	AACTCCTGGG	17160
	CTCAAGTAAT	CCTCCCACCT	TGGCCTCCCA	AAGTGCTGGG	17200
	ATTACAGGCA	TGAGCCACCG	CGCCCGGCCC	TTTTACATTT	17240
	ATTTATTTAT	TTTTTGAGAC	AGAGTCTTGC	TCTGTCACCC	17280
10	AGGCTGGAGT	GCAGTGGCGC	GATCTCGGCT	CACTGCAAGC	17320
	TCTGCCTTCC	AGG TTCACAC	CATTCTCCTG	CCTCGACCTC	17360
	CCGAGTAGCT	GGGACTACAG	GCGCCCGCCA	CTGCGCCCTA	17400
	CTAATTTTTT	GTATTTTTTAG	TAGAGACGGG	GTTTCACCGT	17440
15	GGTCTCGATC	TCCTGACCTC	GTGATCCACC	CGCCTCAGCC	17480
	TCCCAAAGTG	CTGGGATTAC	AGGCGTGAGC	CACTGCGCCC	17520
	GGCCCTTTTA	CATTTATTTT	TAAATTAAGA	GACAGGGTGT	17560
	CACTATGATG	CCGAGGCTGG	TCTCGAACTC	CTGAGCTGAA	17600
20	GTGATCCTCC	CACCTCGGCC	TCCCAAATG	CTGGGATTAC	17640
	CATGTCCAAC	TTTCCACTTC	TTGTTTGACC	AAGGATGGAT	17680
	GGCAGACATC	AGAAGGGGCT	TGGAAAGGGA	GGTGTCAAAG	17720
25	ACCTTGCCCA	GCATGGAGTC	TGGGTCACAG	CTGGGGGAGG	17760
	ATCTGGGAAC	TGTGCTTGCC	TGAAGCTTAC	CTGCTTGTCA	17800
	TCAAATCCAA	GGCAAGGCGT	GAATGTCTAT	AGAGTGAGAG	17840
	ACTTGTTGGAG	ACAGAAGAGC	AGAGAGGGAG	GAAGAATGAA	17880
30	CACTGGGTCT	GTTTGGGGCT	TTCCCAGCTT	TTGAGTCAGA	17920
	CAAGATTTAT	TTATTTATTT	AAGATGGAGT	CTCATTCTGT	17960
	TGCCCAGGCT	GGAGTGCAGT	GGTGCCATCT	TGGCTCACTA	18000
	CAGCCTCCCC	ACCTCCCAGG	TTCAAGTGCT	TCTCCTGCCT	18040
35	CAGCCTCCCG	AGTAGTTGGG	ATTACAGGCG	CCCGCCACCA	18080



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	CACCCAGCTA	ATTTTTGTAT	TTTCAGTAGA	GATGGGGTTT	18120
	CGCCATGCTG	GCCAGGCTGT	TCTCGAAAAC	TCCTGACCTC	18160
5	AGATGATCCA	CCCGCCTCGG	CCTCCCACAG	TGCTGGGATT	18200
	ACAGGCGTGA	GCCACTGCGC	TGGCCAAATC	AGACAAGGTT	18240
	TAAATCCCAG	CTCTGCCTGT	ACTAGCTGAG	GAACTCTGCA	18280
	CACATTTTCAT	AACCTTTTCTG	GGCCTACGTT	CTCACCTTTA	18320
10	ACGTGAGGAT	AATATATCTA	CTTCATAGAC	ACCTTTTTTAT	18360
	GTTGTCTCCA	AGTTTTCTAA	CAGCTCTAGT	TCTGTACCCA	18400
	AGACATGGCA	GGTGGCCAAC	GACATCCTTC	TAGGCTGTGG	18440
	TGATGTGTTT	GGAGCTTGTT	CCACGGGTCT	TGTGTGGGGC	18480
15	CAGCCCTGTT	CAGATAAGGC	CTTGTGGGGT	GGCCTGGGGT	18520
	AGGGGGAGGG	GTTGGGCAAA	CTCTCCCTTA	AAACGCTTTG	18560
	TAACCATCTG	AGGCACCAGC	AAGAGCGGCC	CCCGAGCCTG	18600
	GACAAAATCC	AAACGGCTTC	CTACTTCAAG	CACTGATGTC	18640
20	TAGTGAGTGA	AGGAACAGCT	CTGGGTCCAG	GATATTATAG	18680
	GTCACATTAA	ACTAAAGGGG	CTTGGCCATC	AGCTGGCTTC	18720
	CAGAGCGTCA	GCCAGTTACT	TCACCTCTTT	GGCTTTGGCC	18760
25	TGTTTTTCAGC	TACAAGAGGA	CTTAATCCAG	AGGACCTCAG	18800
	AGGTCCTTCC	CAGCTCAGAC	CTTCTTTGAC	TGTCTCCCAG	18840
	AGACACTGCT	GTAGGAGTGC	ACACCAGTTT	ACTTTTCTTT	18880
	CTTTTGTTTT	TGAGATGGAG	TTTCGCTCTT	TTTGCCTAGG	18920
30	CTGGAGTGCT	GTGGTGTGAT	CTCAGCTCAC	TGCAACCTCT	18960
	GGCTCCCAGG	TTCAAGTGAT	TCTCCTGTCT	CTGCCTCCCG	19000
	AGTAGCTGGG	ATTACAGACA	CCCACCACTG	CACCCGGCTA	19040
	GTTTTTGTAT	TTTCAGTAGA	GATGGGGTTT	CGCCATGCTG	19080
35	GCCAGGCTGT	TCTCGAAAAC	TCCTGACCTC	AGATGATCCA	19120

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°		TCCGCCTTGG	CCTCCCAAAG	TGCTGAGATT	ACAGATGTGA	19160
		GGCACCACAC	CCGGCCATTT	TTGTATTTTT	AGTAGAGACG	19200
		GGGTTTTGCC	ATGTTGGCCA	CGCTGGTCTC	AAACTCCTGA	19240
5		CCTCAAGTGA	TCTGCCCACC	TTGGCCTCCT	GAAGGGCTGG	19280
		GACTACAGGC	GTGAGTCACC	GTGCCC GGCC	ATTTTTGTAT	19320
		TTTTAGGACA	GCGTTTTTTC	ATGTTGGCCA	GGCTGGTCTC	19360
10		AAACTCCTGA	CCTCAAGTGA	TCCACCCACC	CCGGCCTCCC	19400
		AATATGCTGG	GATTCCAGGT	GTGAGTTACC	ATGCCC GGCT	19440
		ACCACTTTAC	TTTTCCTGCA	GGCTATCACA	GAACGTGTAC	19480
		AATCTAGACT	CTAATCAACC	AAATCAACGT	CTTGCCATCG	19520
15		GAGTTTGCTG	GTGAAGGGCA	CTTGGGGTCC	TGGAAATAAC	19560
		TGTAGGCTCC	AAGCCACACA	CACTGAGATA	GGCCTATTCC	19600
		CTGAGGCCTC	AGAGCCCCTG	ACAGCTAAGC	TCCCTTGAGT	19640
		CGGGCAATTT	TCAACAACGT	GCTCTGGGGA	CACAGCATGG	19680
20		CGCCACTGTC	TTTCTGGTCT	CCTGGGGCTC	AGACTATGTC	19720
		ATACACTTCT	TTCCAGGGCA	GTGGGTAACA	AAGTTTGACT	19760
		CCAGAAAGAC	TTCCCTCGAG	GATTTCTACT	TGGATGAAGA	19800
		GAGGACCGTG	AGGGTCCCCA	TGATGTCGGA	CCCTAAGGCT	19840
25		GTTTTACGCT	ATGGCTTGGA	TTCAGATCTC	AGCTGCAAGG	19880
		TCTGTGGGGA	TAGGGGCAGG	GTGGGGGGTG	GATGGAGGGA	19920
		GAGGATAGAG	AAGCAAAACA	GGGTAGTGGG	AATAAAATGA	19960
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30		TGACTTTGAG	CAGGTTAATA	ACATGTCTGA	GCTTTCCTCC	20040
		TCTTAAGATG	GGGCAGGGGA	TCGTTACCAA	CACTTACCCT	20080
		CCCAGGGTTT	GTTGTAAGGA	CGAATAAGGT	AATAGGAAAT	20120
35		GGGCCCTCAG	CACTGGGCAC	CCACATGTTT	GTTCTCTTGA	20160

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5	GACTCCTATT TCTAGAATTT AAAGCCAAAC TTTGAAAAAT	20200
	AATGACAAAC TCCAAATCGT TGGCATCTTT TTTTTTTTTT	20240
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	CACGATCTCG GCTCACCACA ACCTCCGCCC CCGCTGGGTT	20320
	AAAGCGATTC TCTTGCCTCA GCCTCCTGAG TAGCTGGGAT	20360
	TACAGGCGTG TGCCTCCATG CCTGGCTAAT TTTATACAGA	20400
10	CGGGGTTTCT CCATGTTGGT CAGGCTGGTC TCAAACCTCC	20440
	AAACTCAGGT GATCCGCCTG CCTCGGTCTC CCAAAACACA	20480
	GGGGATTCCA GGCATGAGCC ACCACGCTTG GCCAATCGTT	20520
	GGCATTCTAA GGCTTTCAGT GTACCTGACT TCTTTTAGTT	20560
15	CTAAGTCTGT AACTGTTAAC CTTTCTTGGG CCACGGCTAT	20600
	CACACGGATC TCTCTGGGAA TCTGACGACA GTGCCTCAAA	20640
	CCCGAGGGAG CACCGCCAGG TGTGCACACA CGTTTCTGTC	20680
	AACGATTTTCG GAGGACTCTT GGGATCCCTG AACACCATCT	20720
20	GTTCCATGGG ACCTTAGGTT AAGAGCCTCT GTTCAAAGGA	20760
	GGCTTTTGCT CTTGGTGGGT GGATGGGGTG AAGTCTCCAA	20800
	GCCCTCTTRC GGSCCCTTCG GTATTCCTAT NCCCCGGTTC	20840
	TGCCCTGTCT TAGTCCAGTG CTCTCTATTT AACAAATGAG	20880
25	CAGTAAATGT ACACCGATGG ACTTTGGGAG ACAATAAAGA	20920
	CCTGATATTC AATTCTAGCT CCTTAAACCA CAGGAGAACA	20960
	TTCTTTCAGC AGACAACTTC AGTTGGTATT AGGCCAAGGT	21000
	AAGAAAGGCC AACAGCATCC TTTTCTGAAG AAACCTCAGG	21040
30	AGATGGCTCT CTGCCAGAAA GCTATAACCT GGAAGGGGAA	21080
	TTGTAAAATA GATGAGGGGC TGGATGAAGG ACGAGACCAG	21120
	GGCCCCGTCA CGGGAGAGGG AAGGCAGCTC CTGGCTGTGT	21160
35	CTGTCCCCCG GCTTTTGGGC TCTGAAGGAC TAACCACATG	21200

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	CTTTCTCACT	TGTCTCAGAT	TGCCCAGCTG	CCCTTGACCG	21240
	GAAGCATGAG	TATCATCTTC	TTCCTGCCCC	TGAAAGTGAC	21280
	CCAGAATTTG	ACCTTGATAG	AGGAGAGCCT	CACCTCCGAG	21320
5	TTCATTTCATG	ACATAGACCG	AGAACTGAAG	ACCGTGCAGG	21360
	CGGTCCTCAC	TGTCCCCAAG	CTGAAGCTGA	GTTACGAAGG	21400
	CGAAGTCACC	AAGTCCCTGC	AGGAGATGAG	TATGTCTGAA	21440
10	GACCCTTTTCG	CTCTTGGTGG	GTGGATGGGG	TGGGGCAGGG	21480
	TCTTTGGGCC	TTCCACTGTG	CTAAGCAGAA	CGCAAGGGCT	21520
	CCACAGGCTT	GTAGGGGGGC	CGTGGATGAG	TCCTTAATCC	21560
	TCATCGTGCC	AGAAGGGAAG	GCTGAACTGC	CTTCTCTCAT	21600
15	CAGACTCATT	CCTCAGCCTC	ACGAGCAGAC	CTCCCTGACA	21640
	GGCGCTCACA	ACACTGCCTC	TCAAGACGAG	TCTGTCTGAC	21680
	CTGTTTTCTC	ATCTTGACCT	AACTTGCTAA	ATGCTCCTGG	21720
	GCAAGTCACT	CCACCCTCGG	TCAGCTCAGA	CCTCTTCAGG	21760
20	CCTCAGAGAA	AGTCAACAGT	GCTGCGCCAT	CCCAGCTTGC	21800
	TTGCAAAGGG	ATCCCTTGGT	TGGGGTGTTG	GGGAAGGCAG	21840
	GGTTTTAACG	GAAATCTCTC	TCCATCTCTA	CAGAGCTGCA	21880
	ATCCTTGTTT	GATTCACCAG	ACTTTAGCAA	GATCACAGGC	21920
25	AAACCCATCA	AGCTGACTCA	AGGTGGAACA	CCGGGCTGGC	21960
	TTTGAGTGGA	ACGAGGATGG	GGCGGGAACC	ACCCCAGCC	22000
	CAGGGCTGCA	GCCTGCCCAC	CTCACCTTCC	CGCTGGACTA	22040
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30	GACACAGGGG	CCCTTCTCTT	CATTGGCAAG	ATTCTGGACC	22120
	CCAGGGGCCC	CTAATATCCC	AGTTTAATAT	TCCAATACCC	22160
	TAGAAGAAAA	CCCGAGGGAC	AGCAGATTCC	ACAGGACACG	22200
35	AAGGCTGCCC	CTGTAAGGTT	TCAATGCATA	CAATAAAAGA	22240

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GCTTTATCCC	TAACTTCTGT	TACTTCGTTC	CTCCTCCTAT	22280
TTTGAGCTAT	GCGAAATATC	ATATGAAGAG	AAACAGCTCT	22320
TGAGGAATTT	GGTGGTCCTC	TACTTCTAGC	CTGGTTTTAT	22360
CTAAACACTG	CAGGAAGTCA	CCGTTCATAA	GAACCTCTTAG	22400
TTACCTGTGT	TGGATAAGGC	ACGGACAGCT	TCTCTGCTCT	22440
GGGGGTATTT	CTGTACTAGG	ATCAGTGATC	CTCCCGGGAG	22480
G				22481

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CLAIMS

1. A method of enhancing neuron cell survival comprising:
  - treating a cell population comprising neurons with an effective amount of pigment epithelium - derived factor; and
  - enhancing neuronal cell survival in said population.
2. A method of inhibiting glial cell proliferation comprising:
  - treating a cell population in comprising glial cells with an effective amount of pigment epithelium derived factor; and
  - inhibiting glial cell proliferation in said population.
3. The method according to claim 1 wherein the neuronal cells are in a tissue cell culture.
4. The method according to claim 1 further comprising:
  - setting up a cell culture; and
  - treating said cell culture with an effective amount of PEDF.
5. The method according to claim 1, wherein the cells treated comprise a component of tissue being transplanted into a subject.
6. The method according to claim 6, wherein the cells are fetal brain cells.
7. The method according to claim 2, wherein the glial cells are part of a tumor growth.

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°                   8.    The method according to claim 2, wherein  
glial cell growth inhibited is a gliosis.

5                   9.    Purified antibodies or antigen-binding  
fragments of said antibodies raised against a purified  
pigment epithelium-derived factor or an antigenic fragment  
thereof.

10                  10.   The isolated antibodies or antibody  
fragments of claim 9, wherein said antibodies are  
polyclonal.

11.   The antibodies or antibody fragments of  
claim 9, wherein said antibodies are monoclonal.

15                  12.   The antibodies or antibody fragments of  
claim 9, wherein said antibodies are labeled with a  
detectable label.

20                  13.   A method of inhibiting pigment epithelium  
derived factor comprising:

treating cells or a population of cells  
with an effective amount of antibody or antigen binding  
fragments of said antibodies of claim 9; and

25                         inhibiting pigment epithelium derived  
factor biological activity.

14.   A method of determining levels of pigment  
epithelium - derived factor in a fluid, cellular or tissue  
sample, said method comprising:

30                         A.   contacting said sample with purified  
antibodies or antigen-binding fragments according to claim  
9 under conditions in which an immune complex forms  
between said antibodies or antigen binding fragments and  
any pigment epithelium-derived factor present in said  
35                         sample;

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B. separating excess antibodies or antigen binding fragments and thereby from immune complexes; and

C. determining the level of immune complexes determining levels of pigment epithelium - derived factor.

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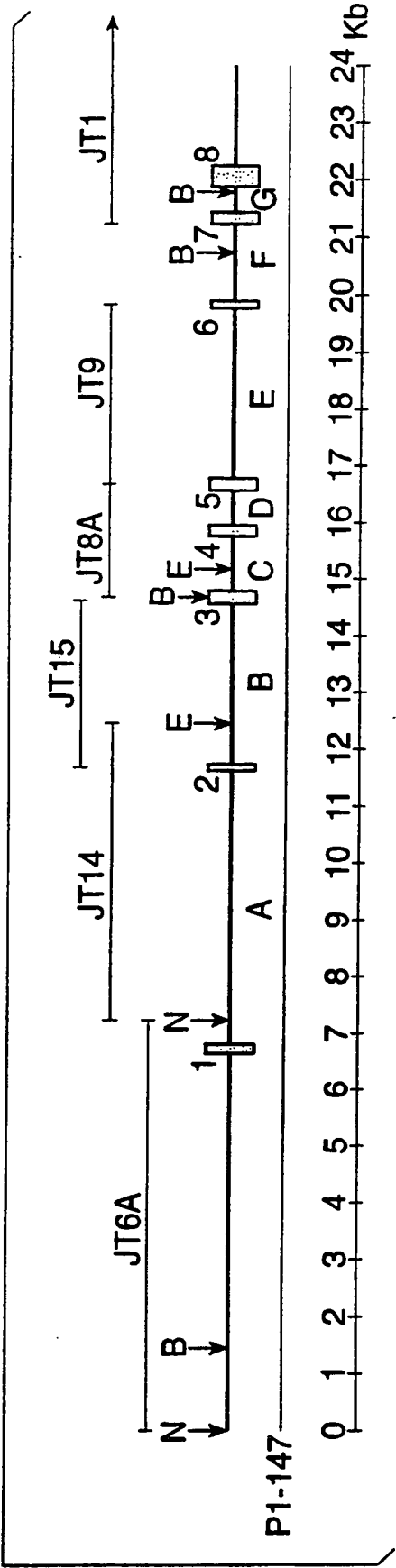
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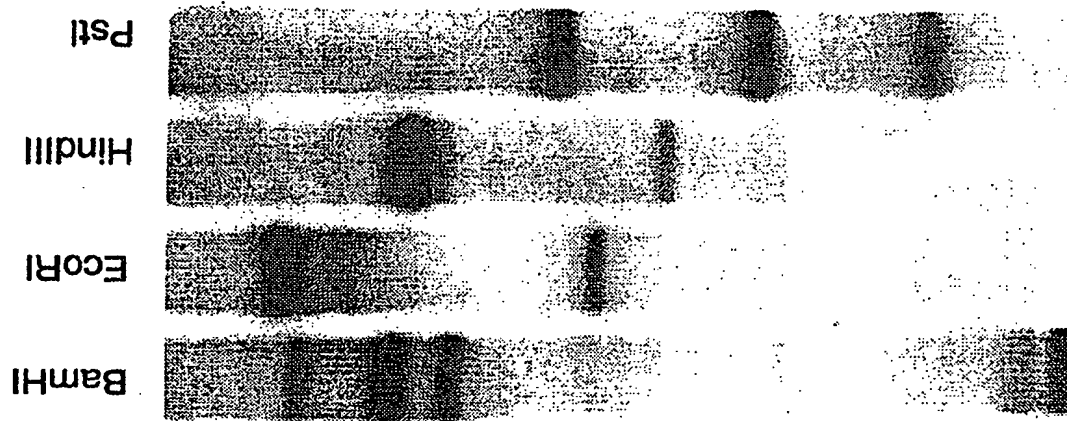


FIG. 1

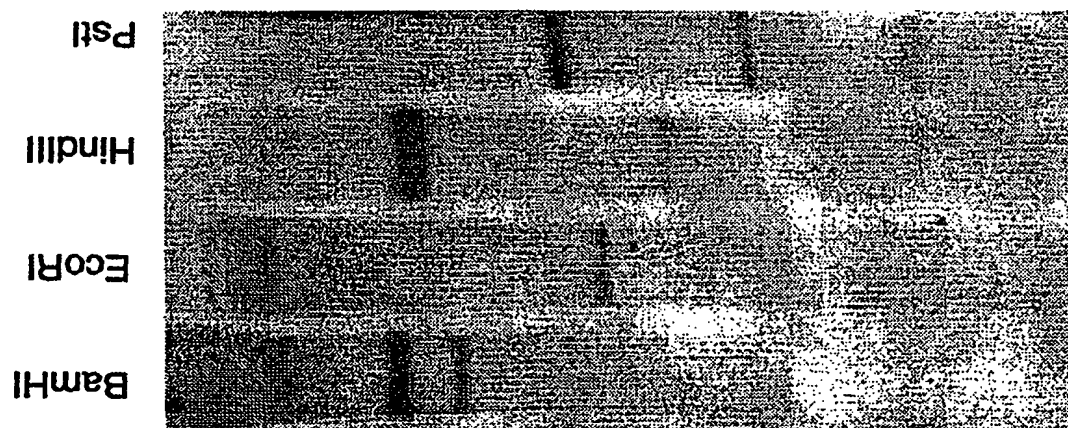


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**FIG. 2B**



**FIG. 2A**



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## FIG. 3

-1050 tgggaggctgaagggggcgggatcacctgaggtcaggagtttgagacaag -1001  
 -1000 cgtgaccaatgtggtgaaacccctgtctctactaaaaatacaaaaattagc -951  
 -950 cgggcatgctcgtgcacacctatagtcaccaactactcagcaggggtgaggc -901  
 -900 aggagaacctcttgaacccgggaagcggaggttgcaqtgaqccgacattg -851  
 -850 cacccctgcactccagccctgggtgacagagtgaagtctccactggaaaaaa -801  
 -800 aaaaaaaaagaacagtgtgatacattgacctaaaggtttaagaacatgcaaa -751  
 -750 ctgatactatatatcacttagggacaaaaacttacatggtaaaagtaaaa -701  
 C/EBP  
 -700 agaaatgtacgaaaaataataaaaaatcaaattcaagatgggtggttatgggtg -651  
 -650 acgggaaagaactgaggcggaatatataagggtgtcactatattgagaaat -601  
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 -300 gccactgcqcccagctttgtttgcatttttaggtgagatgggggtttcacc -251  
 TREP/RAR  
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 -200 tcagttctcccaaagtctggtgattacaggcggttagccctgcgcccggccc -151  
 PEA3 PEA3 PEA3 Oct  
 -150 ctgaaggaaaaatctaaaggaagaggaaggtgtgcaaatgtgtgcgcctta -101  
 HNF-1  
 -100 ggcgtaatgatggtggtgcagcagtggtttaaagtttaacacgagacagtg -51  
 Oct AP-1?  
 -50 atgcaatcacagaatccaaattgagtgaggtcgctttaagaaaggagta -1  
GCTGTAATCTGAAGCCTGCTGGACGCTGGATTAGAAGGCAGCAAAAAAG  
CTCTGTGCTGGCTGGAGCCCCCTCAGTGTGCAGGCTTAGAGGGGACTAGGC  
TGGGTGTGGAGCTGCAGCGTATCCACAG

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FIG. 4C

Heart  
Brain  
Lung  
Liver  
Kidney



Peripheal Blood Leucocyte

FIG. 4B

Spleen  
Thymus  
Prostate  
Testis  
Ovary  
Small Intestine  
Colon



FIG. 4A

Heart  
Brain  
Placenta  
Lung  
Liver  
Skeletal Muscle  
Kidney  
Pancreas



FIG. 5A

Bacteria  
Yeast  
N. matode  
Fly  
Frog  
Sea Urchin  
Clam  
Lobster  
Chicken  
Mouse  
Human  
λ Hind III

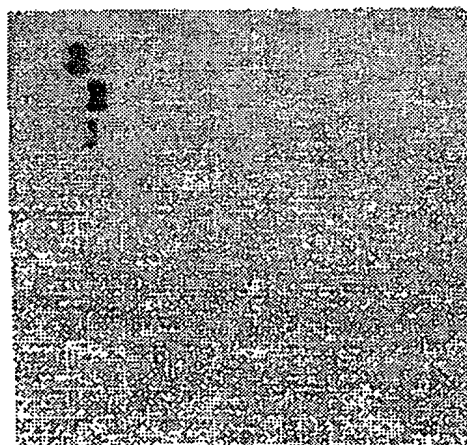


FIG. 5B

Dog  
Cat  
Rabbit  
Cow  
Sheep  
Mouse  
Rat  
Hamster  
Pig  
Marmoset  
Human  
λ Hind III

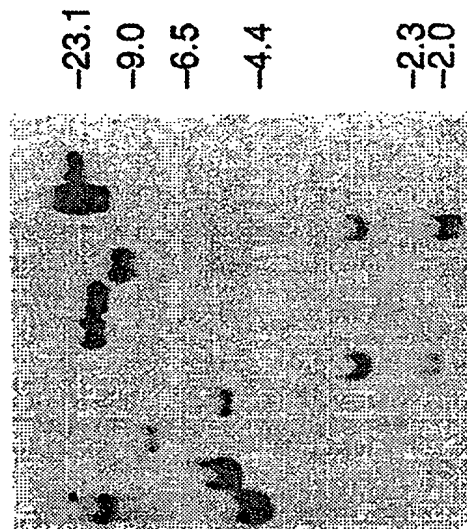
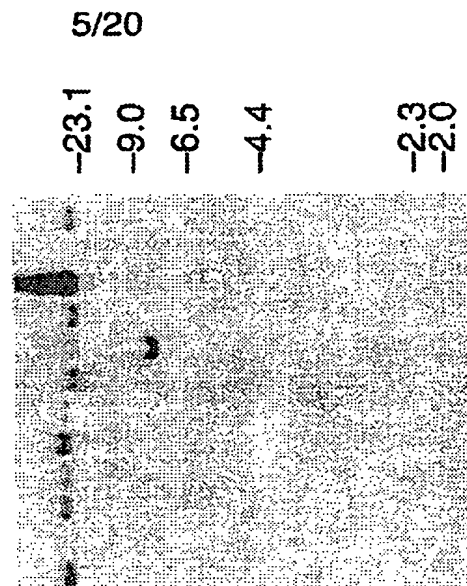
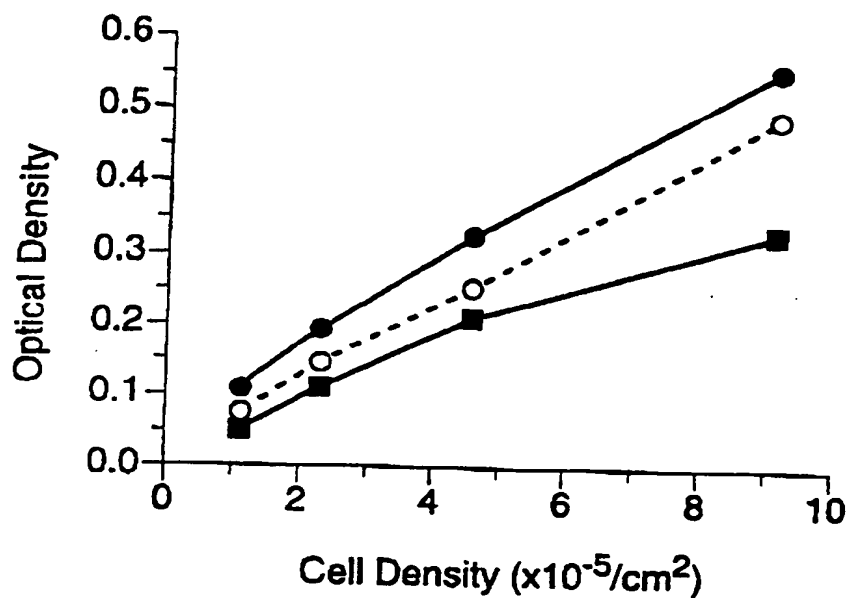
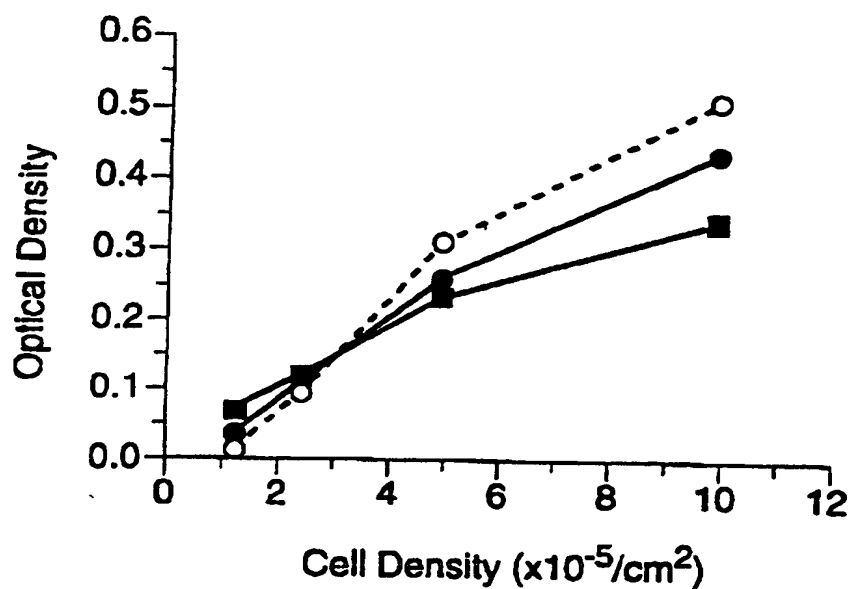


FIG. 5C

Orangutan 1-3  
Common Chimp  
4,5,6,7,8  
Gorilla  
Marmoset  
Macaque  
Human

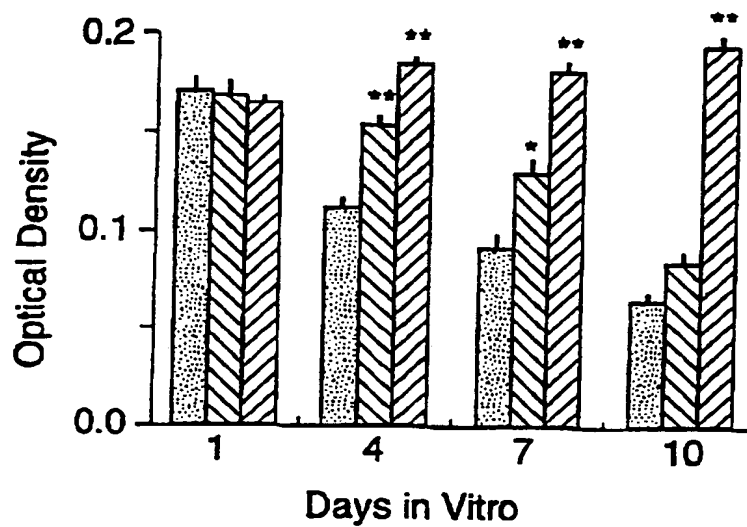
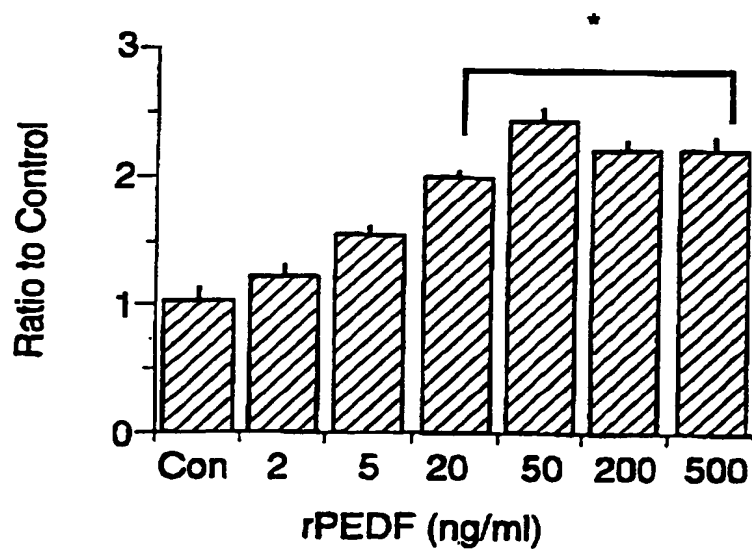


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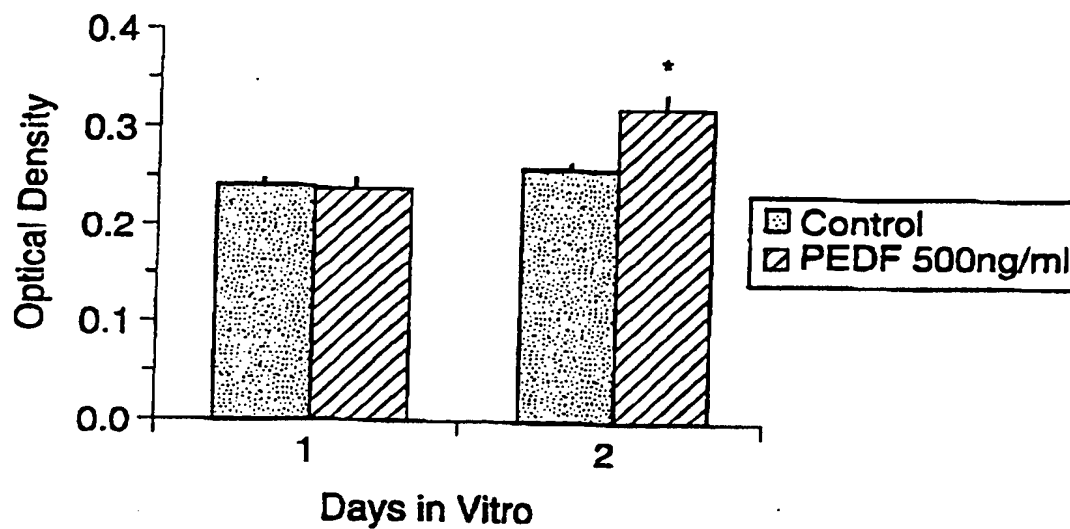
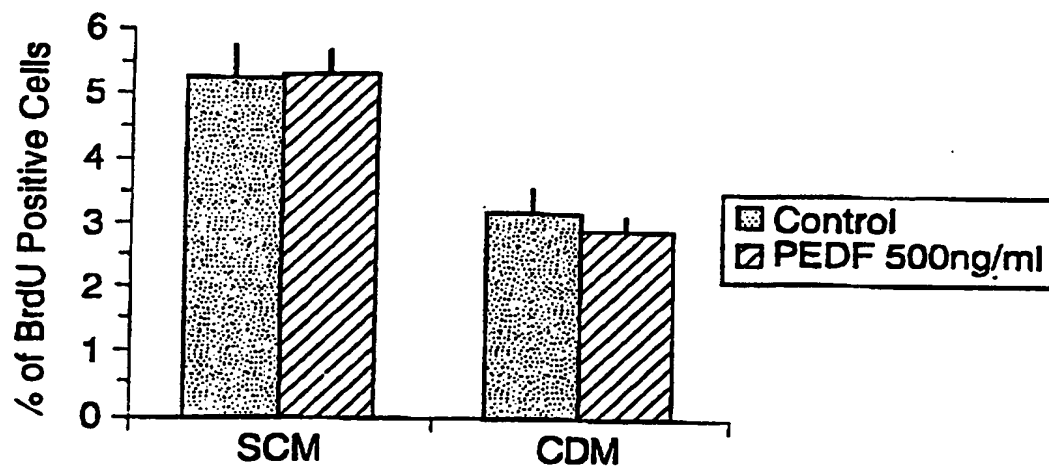
**FIG. 6A****FIG. 6B**

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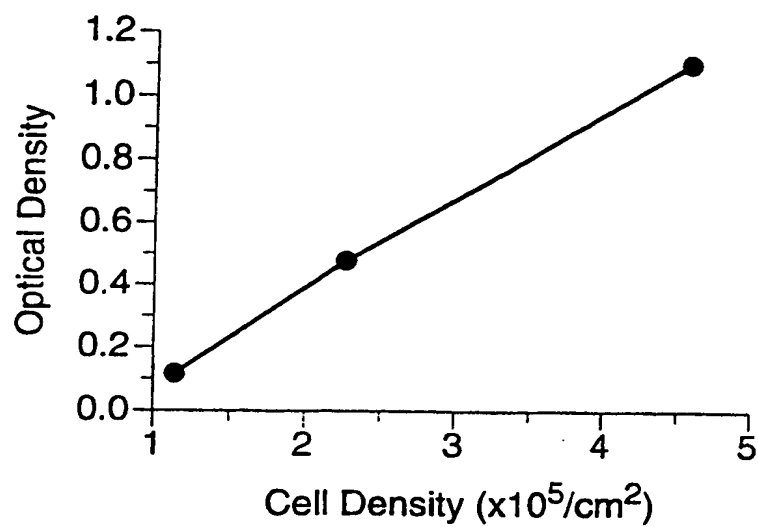
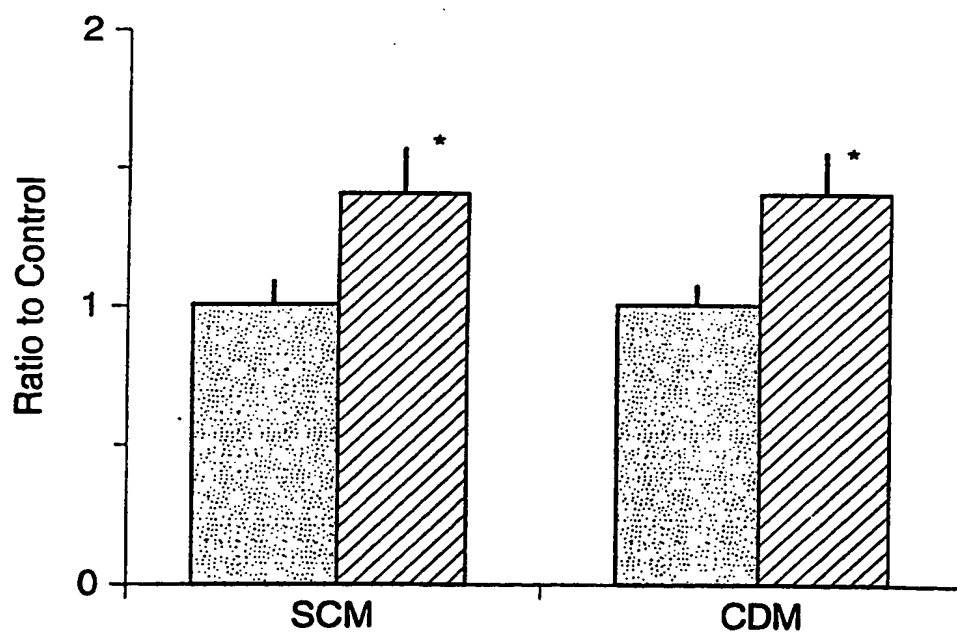
**FIG. 7****FIG. 8**

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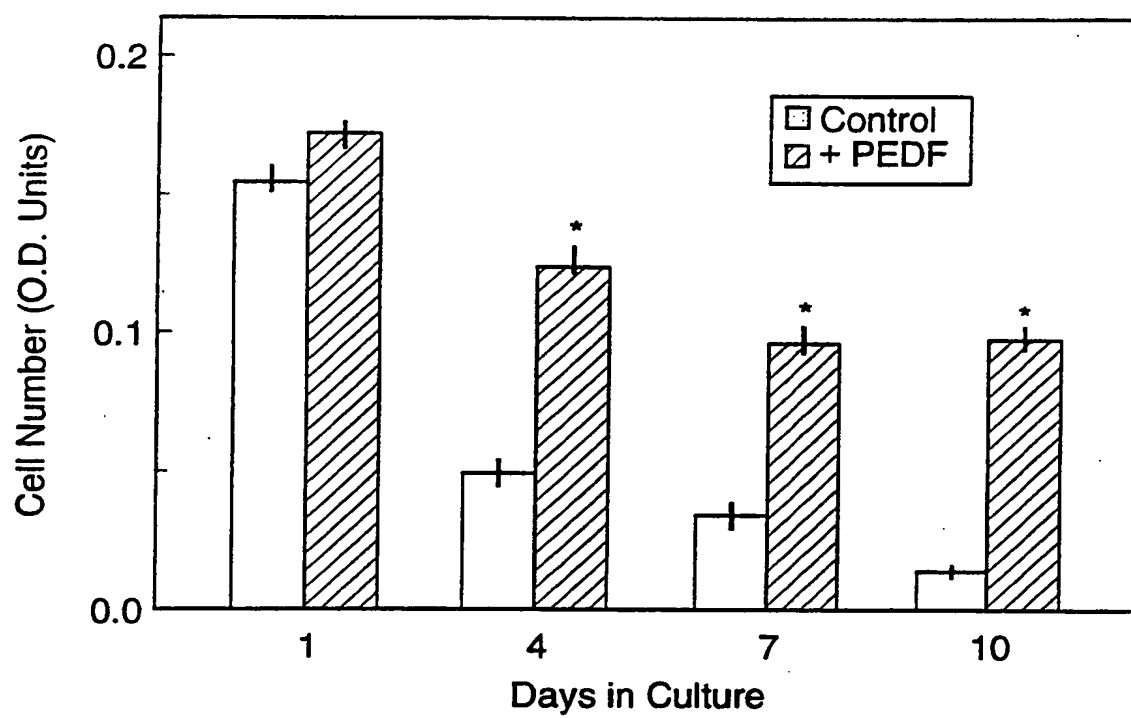
**FIG. 9****FIG. 10**



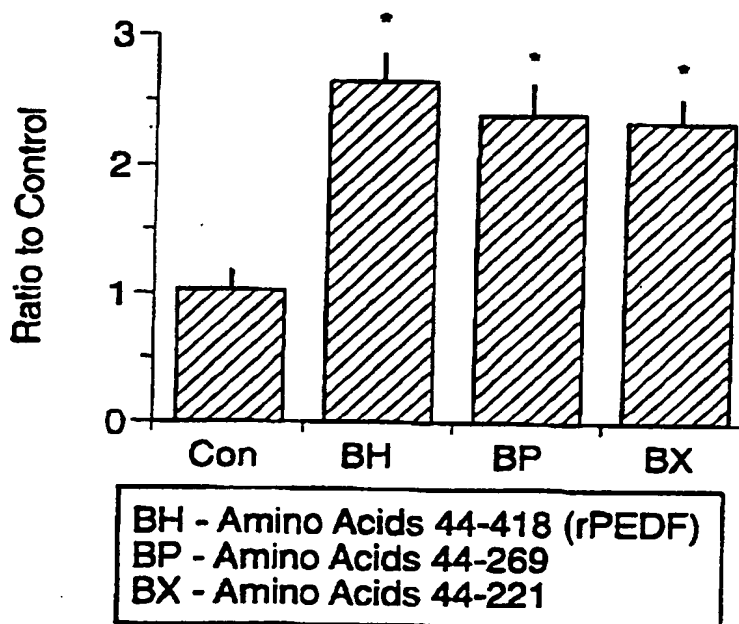
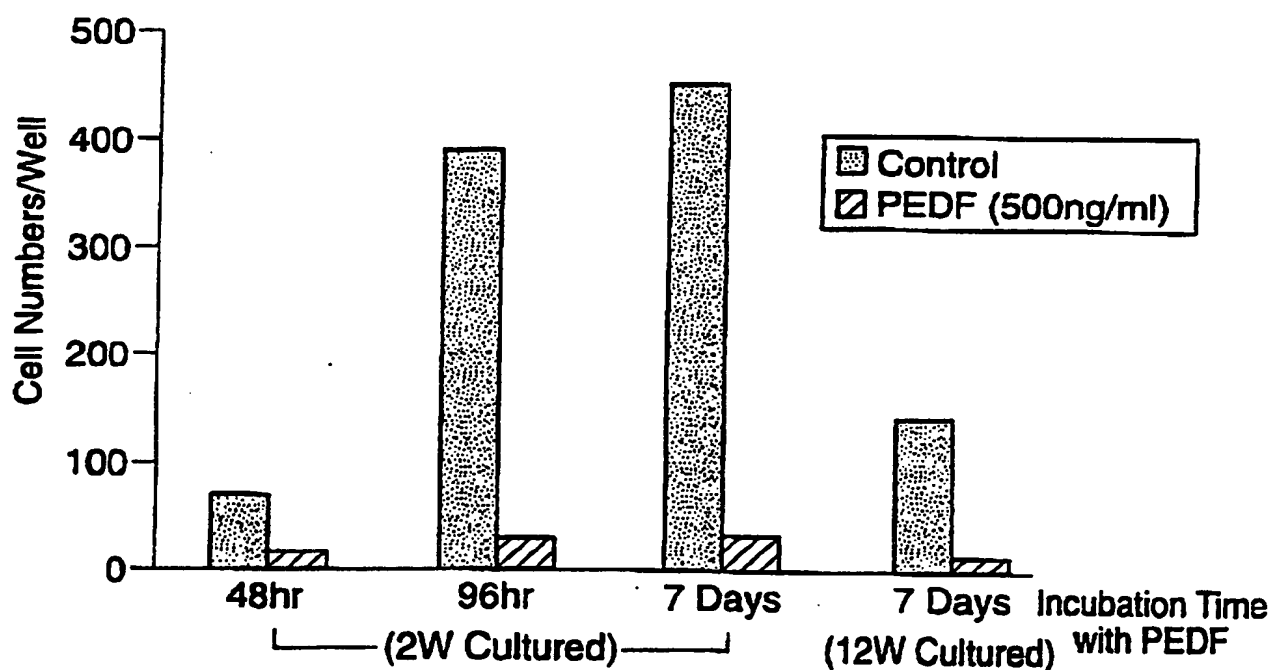
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**FIG. 11****FIG. 12**

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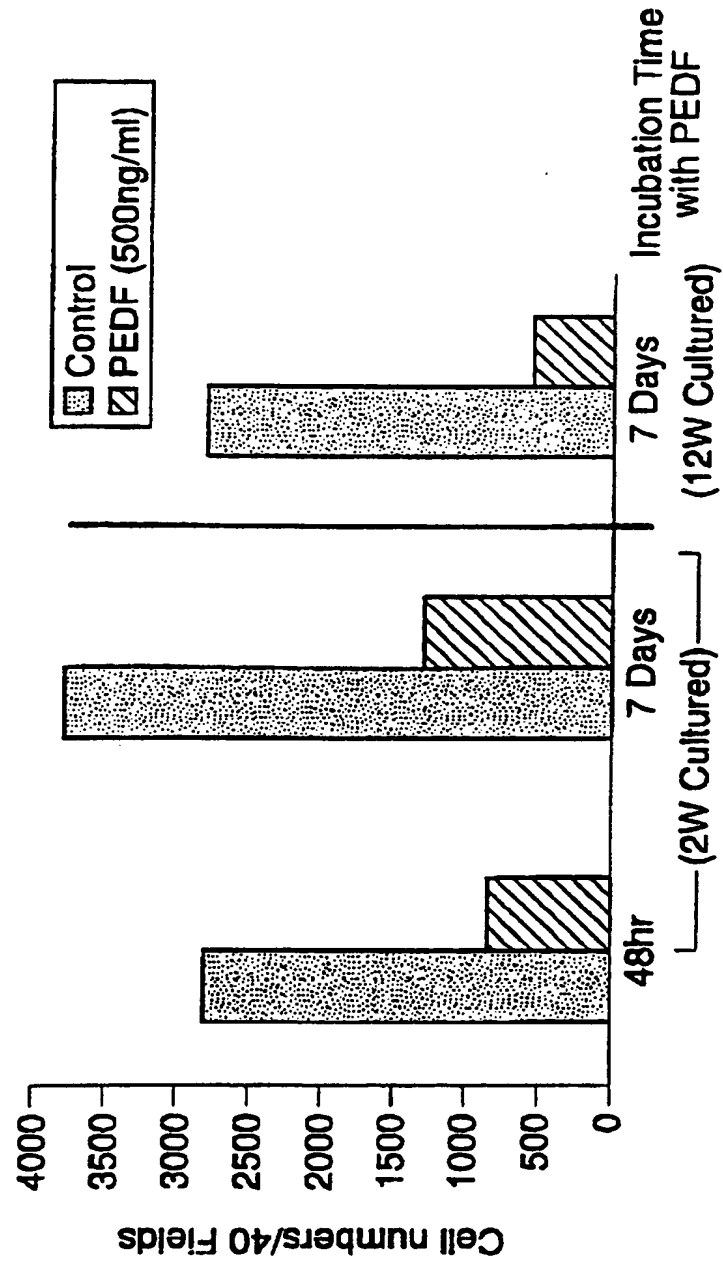
**FIG. 13**

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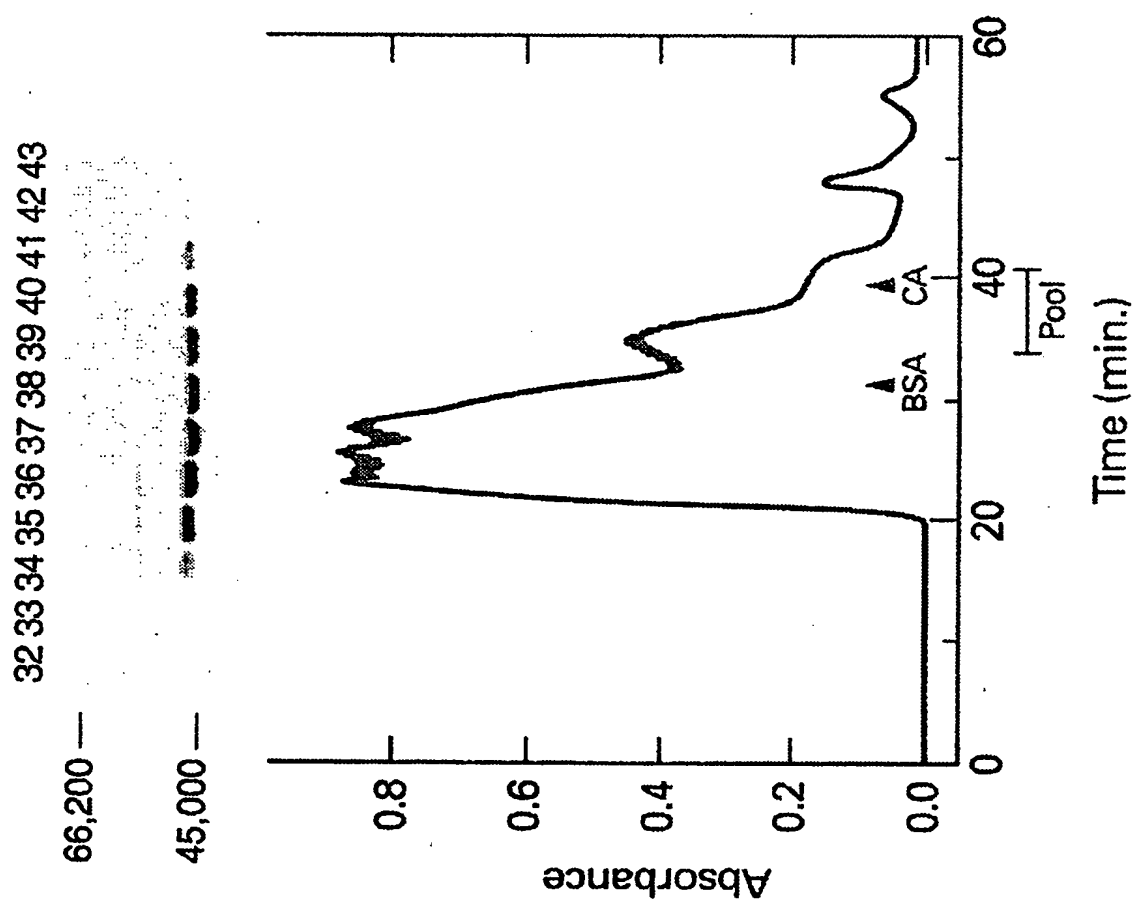
**FIG. 14****FIG. 15**

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**FIG. 16**

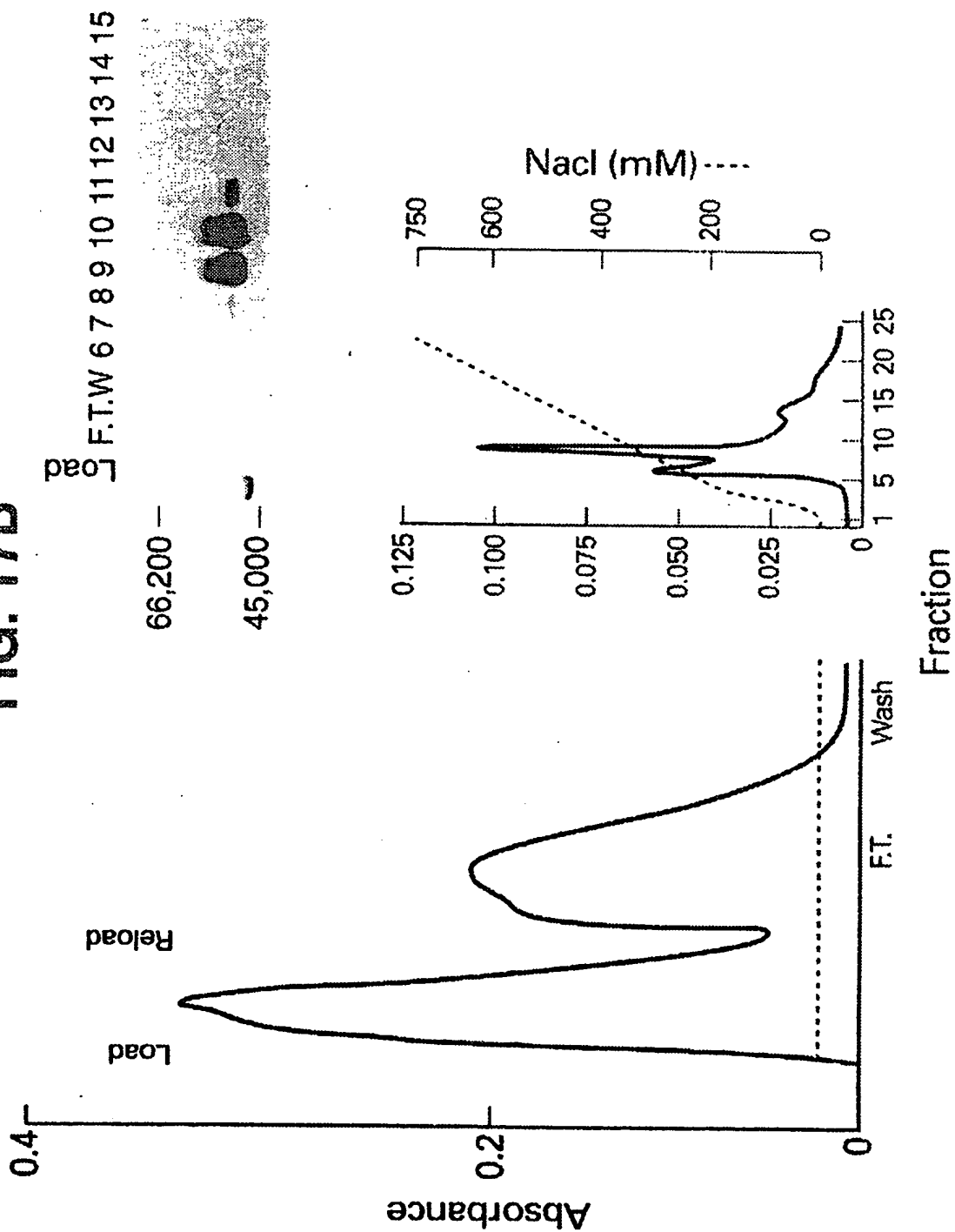


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**FIG. 17A**

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FIG. 17B



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FIG. 18B

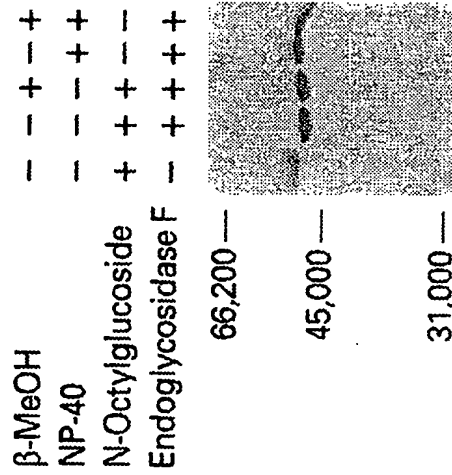
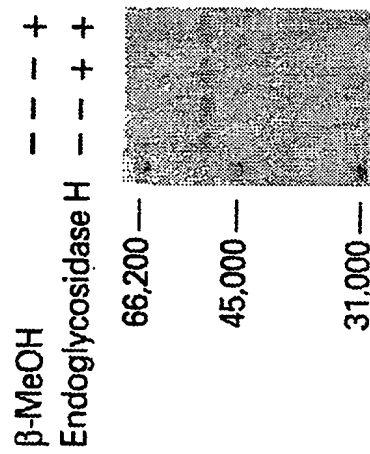


FIG. 18A



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FIG. 19B

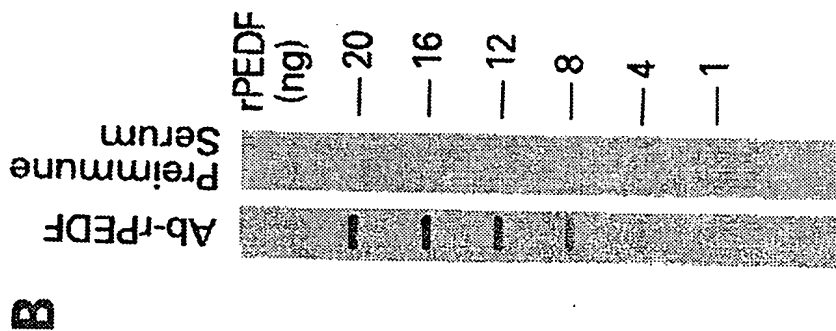
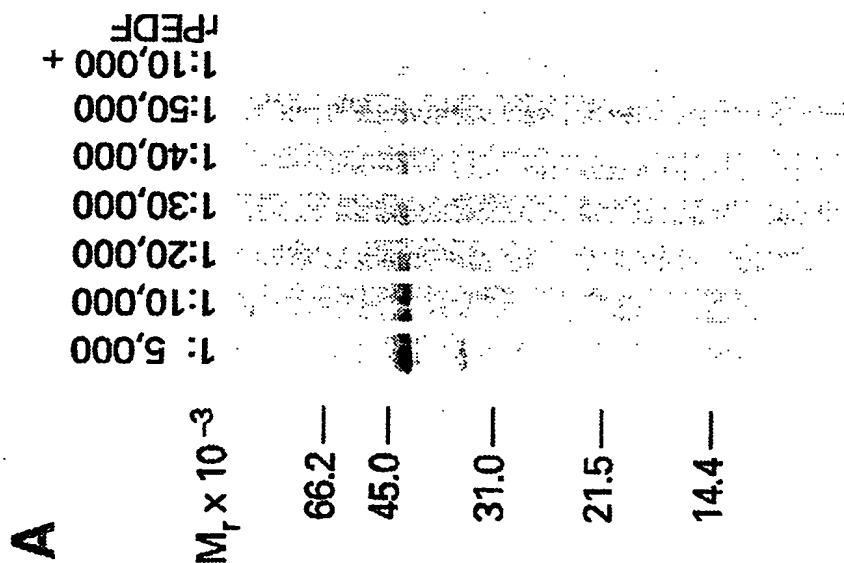
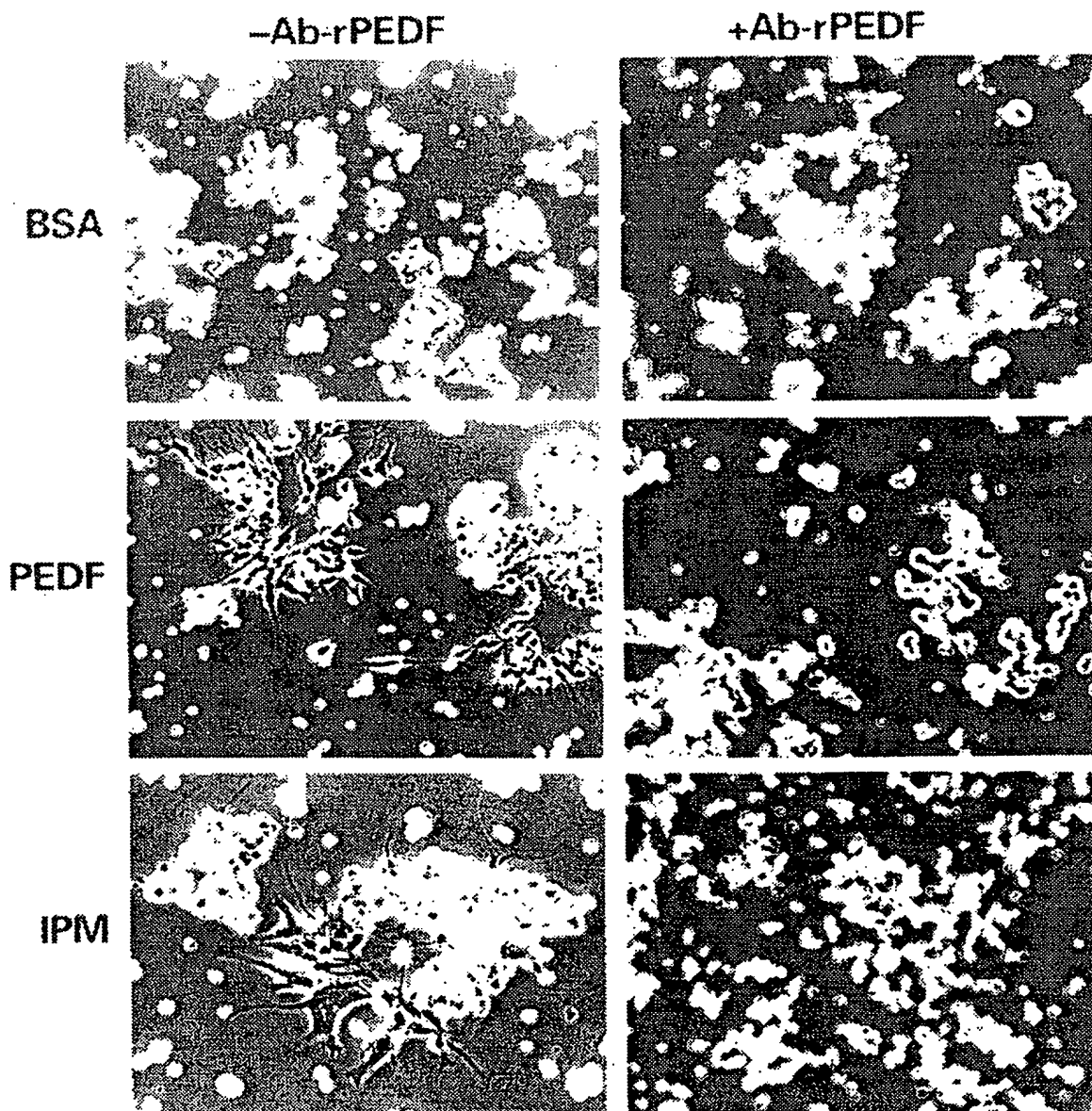


FIG. 19A



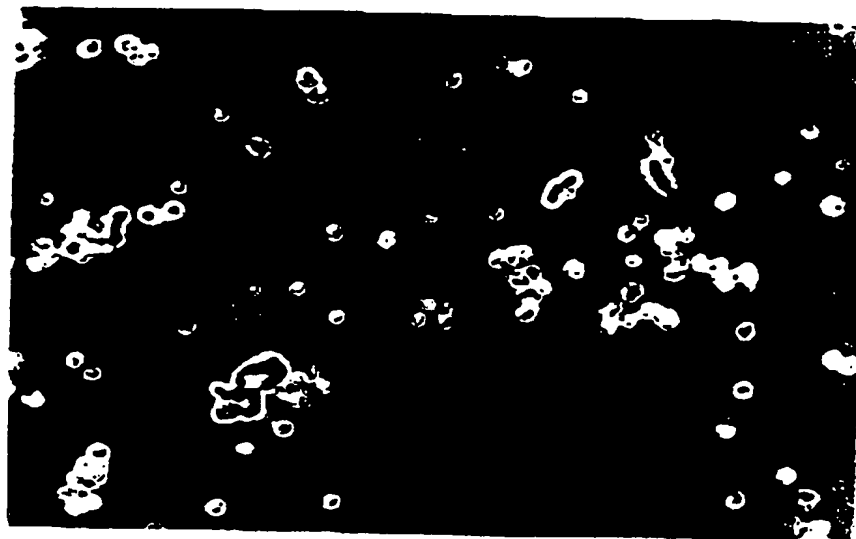


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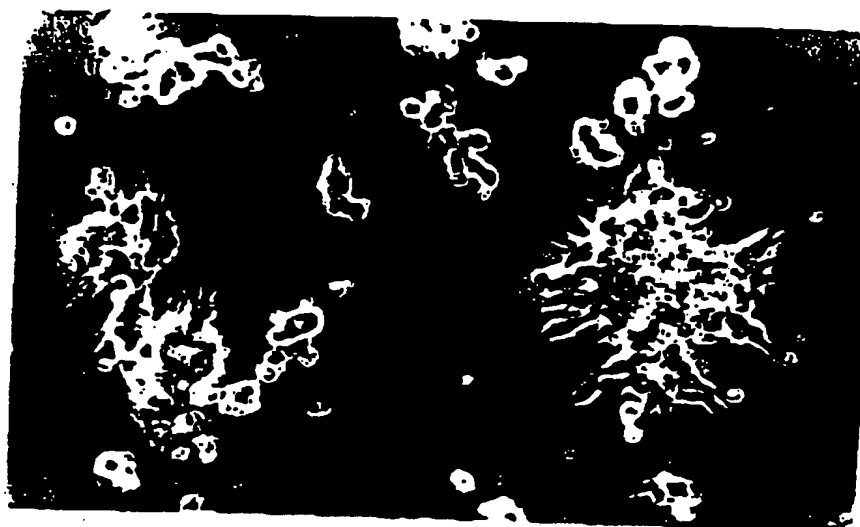
**FIG. 20**

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**FIG. 21A**



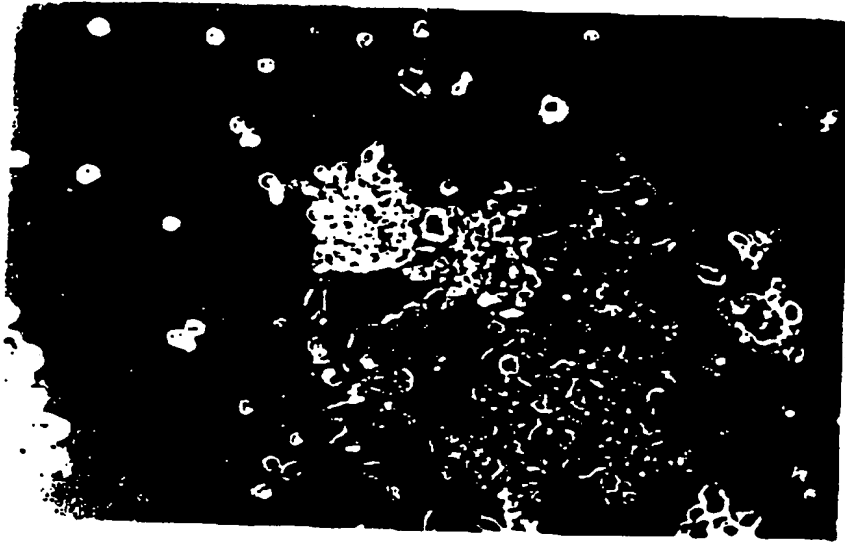
**FIG. 21B**



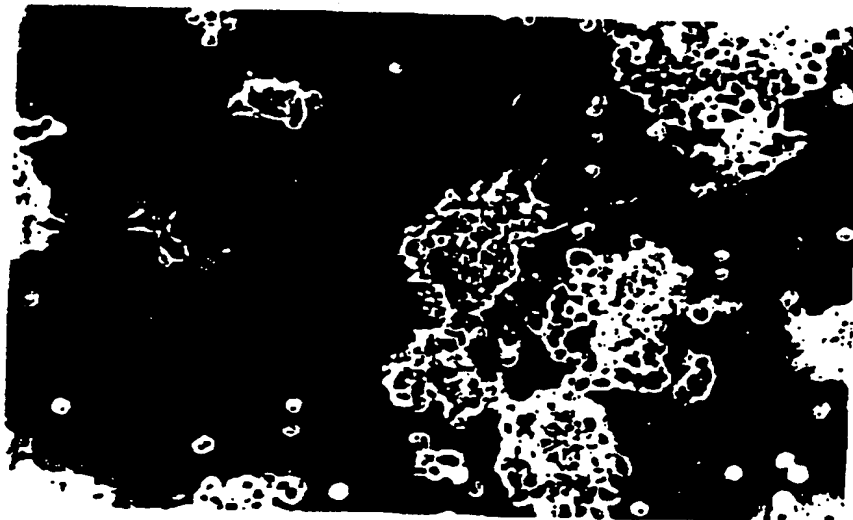
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**FIG. 22A**



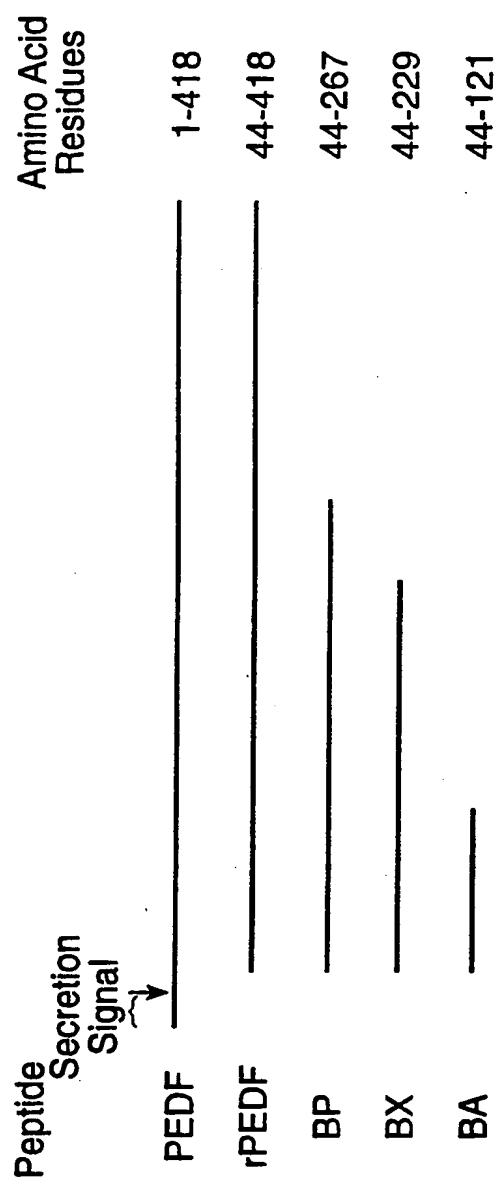
**FIG. 22B**



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FIG. 23



# INTERNATIONAL SEARCH REPORT

Internat. Application No  
PCT/US 95/07201

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K38/57 C07K16/38 G01N33/53 //C07K14/81

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE FASEB JOURNAL, vol. 8, no. 7, 19 April 1994 page A1302 BECERRA ET AL 'PIGMENT EPITHELIUM-DERIVED FACTOR: CHARACTERIZATION USING A HIGHLY SPECIFIC POLYCLONAL ANTIBODY' see abstract 252 ---	9-14
X	WO,A,93 24529 (UNIV SOUTHERN CALIFORNIA) 9 December 1993 see page 5, line 2 - line 30 see page 20, line 19 - page 22, line 26 --- -/--	1,3-6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

3 October 1995

Date of mailing of the international search report

22.11.95

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 268, no. 31, 5 November 1993 pages 23148-23156, BECERRA ET AL 'OVEREXPRESSION OF FETAL HUMAN PIGMENT EPITHELIUM-DERIVED FACTOR IN ESCHERICHIA COLI.A FUNCTIONALLY ACTIVE NEUROTROPHIC FACTOR' see page 23148,abstract ---</p>	
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES,USA, vol. 90, February 1992 pages 1526-1530, STEELE ET AL 'PIGMENT EPITHELIUM-DERIVED FACTOR:NEUROTROPHIC ACTIVITY AND IDENTIFICATION AS A MEMBER OF THE SERINE PROTEASE INHIBITOR GENE FAMILY' see page 1526,abstract ---</p>	
A	<p>DATABASE CHEMICAL ABSTRACTS FILE SERVER STN KARLSRUHE ABSTRACT NO.117:45182, GAUR ET AL 'RPE CONDITIONED MEDIUM STIMULATES PHOTORECEPTOR CELL SURVIVAL,NEURITE OUTGROWTH AND DIFFERENTIATION IN VITRO' &amp; EXP.EYE.RES. (1992) 54 (5),645-59 see abstract ---</p>	
A	<p>DATABASE CHEMICAL ABSTRACTS FILE SERVER STN KARLSRUHE ABSTRACT NO.118:188996, KLIDMAN ET AL 'EFFECTS OF MEDIUM CONDITIONED BY RETINAL PIGMENTED EPITHELIAL CELLS ON NEUROTRANSMITTER PHENOTYPE IN RETINOBLASTOMA CELLS' &amp; CANCER LETT. (SHANNON,IREL.) (1993) 68 (2-3), 207-13 see abstract ---</p>	
P,X	<p>SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 20, no. 1-2, November 1994 page 873 SUGITA ET AL 'EFFECTS OF PIGMENT EPITHELIUM-DERIVED FACTOR (PEDF) ON ASTROCYTES AND MICROGLIA IN CULTURE' see abstract 365.7 -----</p>	1-8

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 95/07201

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1,2,5-8 and 13 partially, in so far as they relate to an in vivo method, are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

### Information on patent family members

**PCT/US 95/07201**

Form PCT/ISA/210 (patent family annex) (July 1992)